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EDITED BY

W. H. PEARSALL
D.Sc., F.R.S.
Quain Professor of Botany
University College, London

ASSISTED BY

F. G. GREGORY, D.Sc., F.R.S.
Professor of Plant Physiology
Imperial College of Science and Technology, London

T. M. HARRIS, M.A., PH.D., F.R.S.
Professor of Botany
The University, Reading

C. T. INGOLD, D.Sc., PH.D.
Professor of Botany
Birkbeck College, London

K. MATHER, D.Sc., F.R.S.
Professor of Genetics
The University, Birmingham

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CONTENTS

PAGE

Ferguson, T. P., and Bond, G. Symbiosis of Leguminous Plants and Nodule Bacteria. V. The Growth of Red Clover at Different Oxygen Tensions. With Plates XIX and XX and two Figures in the Text 385

Wardlaw, C. W. Experimental and Analytical Studies of Pteridophytes. XXVI. *Ophioglossum vulgatum*: Comparative Morphogenesis in Embryos and Induced Buds. With Plate XXI and ten Figures in the Text 397

Holden, H. S. The Morphology of a New Species of Pteridosperm Seed from the Yorkshire Coal Measures. With Plate XXII and seven Figures in the Text 407

Fowden, L. The Nitrogen Metabolism of Groundnut Plants: the Role of γ-methyleneglutamine and γ-methyleneglutamic Acid. With five Figures in the Text 417

Stant, Margaret Y. The Shoot Apex of some Monocotyledons. II. Growth Organization. With three Figures in the Text 441

Allsopp, A. Experimental and Analytical Studies of Pteridophytes. XXIV. Investigations on *Marsilea*. 4. Anatomical Effects of Changes in Sugar Concentration. With Plates XXIII–XXV and three Figures in the Text 449

Pramer, D. The Movement of Chloramphenicol and Streptomycin in Broad Bean and Tomato Plants. With two Figures in the Text 463

Simmonds, N. W. Anthocyanins in Bananas. With one Figure in the Text 471

Walker, F. T. The Laminariaceae off North Shapinsay: Changes from 1947 to 1953. With six Figures in the Text 483

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Symbiosis of Leguminous Plants and Nodule Bacteria

V. The Growth of Red Clover at Different Oxygen Tensions

BY

T. P. FERGUSON[1]

AND

G. BOND

(Botany Department, University of Glasgow)

With Plates XIX and XX and two Figures in the Text

ABSTRACT

A comparison has been made of the effect of oxygen concentration on the growth of nodulated red clover plants dependent on nodule-nitrogen and of non-nodulated plants provided with ammonium-nitrogen. Two techniques have been used, the first providing for the exposure of the root system only to adjusted oxygen supply, while in the second the whole plant was so exposed. Four levels of oxygen were employed, namely, 21, 12, 5, and 1 per cent.

With the first method each step in the reduction of oxygen supply was attended by a significant curtailment of growth in nodulated plants. The responses of the ammonium plants were less marked. It is concluded that clover nodules function best when well supplied with oxygen.

No clear effects of oxygen supply were obtained by the second method, this being due, it is considered, to the severe limitation imposed by the necessary experimental arrangement on the growth of the plants.

INTRODUCTION

INVESTIGATIONS designed to throw light on the effect (direct or indirect) of the oxygen factor on the characteristic activity of leguminous root nodules, namely, fixation of atmospheric nitrogen, have been carried out by Wilson and Fred (1937) and by Bond (1951*a*). In both cases the basic procedure was to compare the responses to variation in oxygen supply shown by plants entirely dependent on nodule-nitrogen and by non-nodulated plants supplied with combined nitrogen. Any distinctive responses characterizing the former plants can then be linked with the special nitrogen nutrition of the plants, and will in all probability be due to effects exerted in the nodules themselves.

Wilson and Fred (loc. cit.), using red clover rooted in sand, grew the plants wholly enclosed in large aspirator-bottles, the atmospheres within these being adjusted to contain different levels of oxygen. Nodulated plants were found to respond similarly to combined nitrogen plants, growth in both being essentially unaffected by reduction of oxygen supply until a partial pressure of 0·05 atm. of oxygen was attained, while below this growth decreased for both types of plant. It was concluded that the nodule has no special requirement for oxygen.

---

[1] Now with Glaxo Laboratories Ltd., Fermentation Research Division, Sefton Park, Stoke Poges, Bucks.

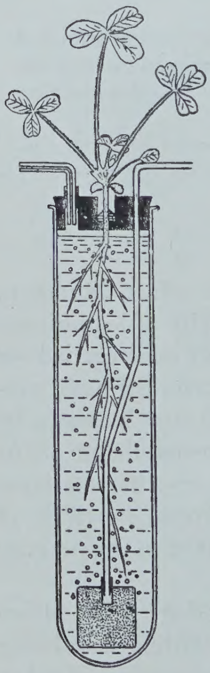
[Annals of Botany, N.S. Vol. XVIII, No. 72, Oct. 1954.]

Bond (loc. cit.) grew Soya-bean plants with their root systems in culture solution maintained in equilibrium with oxygen/nitrogen gas mixtures containing 21, 12, and 5 per cent. oxygen respectively. (Under the prevailing condition of a total gas pressure approximating to 1 atm. the partial pressures of oxygen provided by the gas mixtures would be 0·21, 0·12, and 0·05 atm. respectively.) Nodulated plants showed a progressive reduction in growth, the effects with combined nitrogen plants being much smaller, leading to the conclusion that the nodules have a relatively high oxygen requirement.

In view of the theoretical and practical interest of the oxygen relation of the leguminous root nodule, further work has been carried out, designed to show whether the above divergences in conclusion arose from the use of a different technique or of a different legume. Here both techniques (root-enclosure and whole-plant-enclosure) have been used, with red clover as test plant.

METHODS

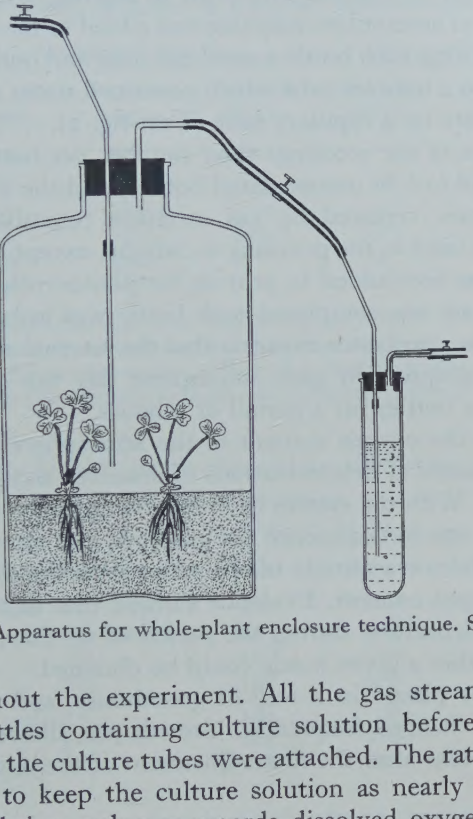
Root-enclosure technique. In principle the procedure resembled that of Bond (1951*a*). Seed of S123 Red Clover (kindly supplied by the Welsh Plant Breeding Station, Aberystwyth) was surface-sterilized and sown in troughs of sterilized sand. After 14 days' growth the seedlings were transferred to water culture in individual test-tubes of 200 c.c. capacity. Each tube was fitted with a three-holed rubber stopper, the largest hole being closed at its base by a rubber inset with a smaller hole through which the root system of the seedling was introduced and the hypocotyl sealed in by means of 'plasticine' (Text-fig. 1, also Pl. XX, Fig. 2). A second hole in the main stopper carried the gas inlet tube, the end of which was fitted to a cubical earthenware diffuser, while the third hole was closed by a small stopper bearing the gas outlet tube made from 1-mm. bore capillary. Crone's nitrogen-free solution (Bond, 1951*b*) was supplied to the plants; this solution was autoclaved before use, and the culture tubes and their fittings were sterilized against the nodule organism. The culture tubes were wrapped in black paper and placed in racks.



TEXT-FIG. 1. Apparatus for root-enclosure technique. See text. ($\times \frac{3}{10}$.)

On the day following transplanting the oxygen content of the culture solution was adjusted to the desired levels (see below), after which certain tubes were inoculated with an effective strain of the nodule organism (Rothamsted strain 49), while other tubes were supplied with ammonium sulphate in an amount ample for growth.

Four levels of oxygen were maintained in the culture tubes. The highest level was obtained by passing air through one series of tubes, and the others by passing oxygen/nitrogen mixtures containing 12, 5, and 1 per cent. oxygen respectively through the other series.[1] The air at first employed was supplied by an Edwards Type IV electrical compressor, ample precautions being taken to free the air-stream from any oil contamination. Later cylinder air was substituted. The oxygen/nitrogen mixtures were obtained in cylinders from the British Oxygen Company Ltd. Except in the case of compressor-air (see later) the air or gas mixtures were bubbled through the culture tubes continuously throughout the experiment. All the gas streams were scrubbed through large bottles containing culture solution before passing into the manifold to which the culture tubes were attached. The rates of gas flow were suitably adjusted to keep the culture solution as nearly as possible at the appropriate equilibrium value as regards dissolved oxygen. The latter was determined by the Winkler process, using the 1-c.c. syringe pipette described by Fox and Wingfield (1938).



TEXT-FIG. 2. Apparatus for whole-plant enclosure technique. See text. (×$\frac{1}{5}$.)

The pH of the culture solution in the tubes was maintained at 6·3 so far as possible. Nodulated plants caused a relatively slight fall in pH, and this was corrected by suitable addition of sodium hydroxide. With plants receiving ammonium sulphate this restoration of pH had to be carried out every 12 hours in the later stages of the experiment. The plasticine seals at the base of the plants were tested daily for freedom from leaks; the culture solution was renewed at fortnightly intervals, and the arrangement of the racks on the greenhouse bench was changed at similar intervals. After growth in water culture had continued for 9 weeks the plants were harvested, individual dry weights and nitrogen contents being determined.

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[1] The results of Wilson (1936) indicate that the variations in nitrogen supply which thus accompanied those in oxygen are unlikely to have had any effect upon growth.

Whole-plant-enclosure technique. The method was that of Wilson and Fred (1937) with certain modifications. Seed (S123) was sown in initially nitrogen-free sand which partly filled 5-litre aspirator bottles and which had been moistened with Crone's solution. In one section of the experiment the seed was inoculated with nodule bacteria prior to sowing, while in the other an adequate amount of ammonium sulphate was added to the sand. Through the rubber stopper closing each bottle passed gas inlet and outlet tubes, the latter being connected to a bubbler tube which contained water and communicated with the atmosphere by a capillary tube (Text-fig. 2).

After emergence of the seedlings their number per bottle was reduced to 7 in inoculated and to 6 in uninoculated bottles, and the air so far present in the bottles was now replaced by gas mixtures resembling in origin and composition those used in the previous technique, except that 1 per cent. of carbon dioxide was now added to provide for photosynthesis. After the displacement of the air was completed each bottle was isolated by closing the three screw-clips in a sequence ensuring that the internal atmosphere was left at atmospheric pressure. On each subsequent day the gas mixtures were passed through the bottles for a period of 2 hours.

Information on the oxygen content of the atmosphere within the bottles was obtained by means of determinations of dissolved oxygen in the water of the bubbler tubes. With the system of intermittent flow indicated above, the routine procedure was to commence the gas-flow, and after a period of 15 to 30 minutes to withdraw a sample of the water with the syringe pipette and determine the oxygen content. Evidence showed that in this way a reliable indication of any departure, during the period of no-flow, from the desired level of oxygen within a given bottle could be obtained.

The bottles were placed in a well-lit greenhouse, and their arrangement on the bench was changed frequently. Growth was allowed to continue for approximately 8 weeks after the first adjustment of the oxygen supply.

RESULTS FROM ROOT-ENCLOSURE TECHNIQUE

Regular determinations of dissolved oxygen in the culture solution showed that during the first half of the growth period the oxygen levels remained at or very near to the equilibrium values appropriate to the different gas streams, but, on account of increased root respiration, they tended to fall after this stage despite increased rates of gas-flow. This tendency was greatest in the 5 and 1 per cent. series, the maximum fall observed being to a level approximately two-thirds of the equilibrium value. The data also showed that oxygen content was uniform at different levels in a given culture tube.

In the nodulated plants the first signs of fixation of nitrogen appeared 3 to 4 weeks after inoculation. A little later differences in growth between the various oxygen treatments began to appear in both nodulated and combined nitrogen plants, and became more marked as the experiment continued. At the same time there was considerable variation in vigour of growth between individual plants at a given oxygen level, due in the main, no doubt, to the genetic differences which are to be expected with a self-sterile type such as clover.

TABLE I

Mean Data (per plant) obtained at Harvest, Root-enclosure Experiments

		Nodulated plants.			Non-nodulated plants.*		
	% oxygen supplied.	No. of plants.	Dry wt. (mg.).	Total N (mg.).	No. of plants.	Dry wt. (mg.).	Total N (mg.).
1953†	21	23	394	—	—	—	—
	12	22	292	—	—	—	—
1951†	21	17	423	12·7	14	693	18·6
	12	18	731	20·4	16	790	21·5
	5	16	492	13·1	14	561	16·5
	1	17	87	2·4	11	168	5·2

* Supplied with a total of 60 mg. N as $(NH_4)_2SO_4$ per plant.

† The 1951 experiment was carried out over the period April to June, that of 1953 over the less favourable period July to September. It is to this that the higher dry weights shown in the 1951 experiment are due. See text regarding the different sources of compressed air in the two experiments.

Minimum differences between means required for significance at $P = 0·05$, *from analysis of variance*

	Comparison.	Dry wt. (mg.). Reqd.	Dry wt. (mg.). Obsvd.	Total N (mg.). Reqd.	Total N (mg.). Obsvd.
Nodulated	21 and 12% (1953)	100	102	—	—
	21 ,, 12% (1951)	144	208	4·0	7·7
	12 ,, 5%	146	239	4·0	7·3
	5 ,, 1%	148	405	4·1	10·7
Non-nodulated	21 and 12%	184	97	4·2	2·9
	12 ,, 5%	184	229	4·2	5·0
	5 ,, 1%	213	393	4·9	11·3

The data obtained at harvest and the results of their statistical treatment are summarized in Table I. The nodulated plants will be considered first. It will be seen that in the main (1951) experiment maximum growth (dry weight basis) in these plants was shown by the 12 per cent. oxygen series. Increase or decrease in oxygen supply from this level resulted in significantly reduced growth, confirming the finding of a preliminary experiment which will not be reported in detail.

That growth at 21 per cent. oxygen should be inferior to that at 12 per cent. was surprising, but was at first accepted as a bona fide result. Later it was decided to reinvestigate the matter, since in the preliminary and 1951 experiments the air-stream was provided by an electrical compressor (as noted already), and this was switched off for 4 hours each night to avoid excessive wear; it seemed possible that this nightly interruption in oxygen supply was responsible for the reduced growth. In 1953 the growth of nodulated plants supplied continuously with air from cylinders was compared with that of plants receiving 12 per cent. oxygen. As shown in Table I, the air plants now grew significantly better than those at 12 per cent. oxygen. Although there may be room for further investigation of this matter, present evidence points to the 1953 result being the correct one, and the results previously obtained with compressor-air will be ignored. On the basis of the 1953 and the remaining 1951 data, the conclusion is that each step in the reduction of oxygen from 21 per cent. is attended by a significant curtailment in the growth of nodulated plants. At $P = 0{\cdot}01$ the differences in means are still significant except for that between 21 and 12 per cent. Similar conclusions are reached from a consideration of the data for fixation of nitrogen, in so far as they are available. Photographs of typical nodulated plants are provided in Pl. XIX, Figs. 1–5, and Pl. XX, Fig. 1.

Control plants (non-nodulated and without added combined nitrogen) set up at each oxygen level showed negligible growth and a mean nitrogen content of 0·1 mg. per plant at harvest, confirming that the conditions precluded access to unintended sources of combined nitrogen. Thus the data in Table I for nitrogen content of nodulated plants may be accepted as indicating nitrogen fixed.

The data for the non-nodulated plants supplied with combined nitrogen (Table I) show that in the 1951 experiment the mean dry weight at 21 per cent. oxygen was lower than at 12 per cent., but the difference did not attain significance. Further reduction to 5 and then to 1 per cent. oxygen resulted in significant curtailment of growth. These findings confirmed the results of a preliminary experiment. It has not been possible to extend the reinvestigation of the effect of the continuity of the air supply to non-nodulated plants, but since they were clearly much less handicapped by the nightly interruption in air-flow than were the corresponding nodulated plants, it is probable that their response to continuous supply would also be smaller. Bearing in mind that the greater growth of nodulated plants supplied with cylinder air as compared with plants receiving 12 per cent. oxygen (1953 experiment) was only just significant, it can with confidence be assumed that any such effect with non-nodulated plants would fail to reach significance.

At $P = 0{\cdot}01$ only the final reduction of oxygen from 5 to 1 per cent. had a significant effect upon mean dry weight. Photographs of combined nitrogen plants are provided in Pl. XIX, Figs. 6–8, and Pl. XX, Fig. 3.

RESULTS FROM WHOLE-PLANT ENCLOSURE TECHNIQUE

Frequent determinations of oxygen content provided no instance of any material deviation from the equilibrium values appropriate to the gas mixtures employed, showing that photosynthesis, during the periods of no-flow, did not increase the oxygen content of the bottles to any significant extent.

As the experiment proceeded it became obvious that the responses to the different oxygen treatments were going to be much less marked than with the previous method, and also that the issue was likely to be obscured by appreciable differences in vigour of growth between the replicate aspirator-bottles at a given oxygen level, the only obvious source of which lay in a fortuitous predominance in certain bottles of plants of genetically more vigorous type.

TABLE II

*Mean Dry-weight Data obtained at Harvest, Whole-plant enclosure Experiment**

% oxygen supplied.	Nodulated plants.	Non-nodulated plants.†
21	0·766 (0·538–1·005)	0·807 (0·370–1·389)
12	0·942 (0·597–1·493)	0·887 (0·531–1·233)
5	0·900 (0·659–1·020)	0·668 (0·438–1·049)
1	0·794 (0·551–0·964)	0·510 (0·386–0·714)

* The data in roman type are mean dry weights (g.) per aspirator-bottle containing 7 plants (nodulated series) or 6 plants (non-nodulated series). There were 6 bottles of each type of plant at each oxygen level. The figures in parentheses show the lowest and highest dry weights recorded for individual bottles.

† Supplied with ammonium sulphate.

The data obtained at harvest are summarized in Table II, and Pl. XX, Fig. 4, shows photographs of bottles selected as typical by eye-judgement. Although certain differences between mean dry weights appear in the table, an analysis of variance of the data shows F values of 0·7 for the nodulated and 1·8 for the combined nitrogen series, compared with a required value of 3·1 ($P = 0{\cdot}05$). Thus the experiment, although carried out on a considerable scale, provides no clear evidence that the growth of the plants was affected by the variations in oxygen supply, and no firm conclusions can be drawn. The plants were actually harvested individually, and statistical treatment of individual plant dry weight data indicated significance for the larger differences between means for the various oxygen levels. In view, however, of the fact that owing to competition the conditions of growth within a given bottle varied somewhat from plant to plant, it is preferable to consider dry weight per bottle.

There is a suggestion that the growth of nodulated plants was not retarded by decreasing oxygen supply until the 5 per cent. level had been passed, in apparent agreement with Wilson and Fred's conclusion (see Introduction), but this does not hold for the combined nitrogen plants. There is also a suggestion of better growth of nodulated plants at 12 per cent. oxygen than at 21 per cent., as was found in the first root-enclosure experiments, but on present evidence this can only be regarded as a coincidence without significance.

The reasons for the absence of a clear response to oxygen treatment will be considered in the following section. A noteworthy effect observed in the experiment was that in the 1 per cent. oxygen bottles, especially those containing nodulated plants, many roots grew out of the sand and projected into the gaseous phase, as shown in Pl. XX, Fig. 5. This was presumably the result of an aerotropic reaction.

DISCUSSION

As already indicated, this investigation was designed to ascertain if the plants and especially the nodules of different legume species vary in their oxygen relation, and if the conclusions are similar with a given legume species irrespective of which of the two techniques is used.

Data obtained by the root-enclosure technique are now available for two legumes, namely, Soya-bean (Bond, 1951*a*) and clover (present paper). Those for clover can be summarized in two ways. Firstly, if the various groups of plants are designated by the percentage of oxygen supplied to their roots, then the statistically significant effects of oxygen treatment on the dry weight of the plants can be shown thus:

$$P = 0{\cdot}05\begin{cases}\text{Nodulated plants:} & 21\% > 12\% > 5\% > 1\%\\ \text{Combined nitrogen plants:} & 21\% = 12\% > 5\% > 1\%\end{cases}$$

$$P = 0{\cdot}01\begin{cases}\text{Nodulated plants:} & 21\% = 12\% > 5\% > 1\%\\ \text{Combined nitrogen plants:} & 21\% = 12\% = 5\% > 1\%\end{cases}$$

Alternatively the results can be expressed on a relative basis as in Table III, data for Soya-bean being included for comparison. From the clover data, summarized in either way, it is clear that the nodulated plants which are dependent on nodule nitrogen show a higher susceptibility to reduction of oxygen supply than do the combined nitrogen plants. Table III shows that in general the results with clover resemble those for Soya bean. Nodulated clover plants show somewhat greater oxygen responses than do the corresponding Soya-bean plants, but since the same holds also for the combined nitrogen plants, the oxygen effect upon the nodules themselves is probably of the same order in the two species.

The same explanation is offered for these clover results as was previously provided for those relating to Soya-bean (Bond, 1951*a*). Attention was directed primarily to the effect of oxygen curtailment on the respiration of legume nodules, a process with which fixation may be closely linked. It was pointed out that while some investigations have indicated a greater rate of respiration (in air) on the part of nodules than on that of roots, others have led to the conclusion that although nodules are potentially more rapidly respiring, oxygen-shortage within the tissues limits the respiration even when the nodules are lying in air, and some degree of anaerobiosis already exists. In either event, reduction of external oxygen supply will be likely to affect nodule respiration before that of roots, so that nodulated plants may be affected before combined nitrogen plants. Implicit in this explanation of the present results is that a reduction in respiration or an increase in anaerobiosis in the nodule will curtail fixation, for which no direct evidence exists.

It is clear from the previous section that in the present authors' experience the two techniques, used with the same legume, did not give the same result. The absence of a clear result in the whole-plant-enclosure experiment is not interpreted as meaning that when the whole plant is involved a reduction from 21 to 1 per cent. oxygen is without effect. Actually, exposure of the whole plant rather than the root system only to reduced oxygen level would be expected to have a more drastic effect. The lack of clear response is attributed to a limitation of growth which is almost inherent to the method and which is obvious from the data. Thus while the dry weight of plants supplied with 1 per cent. oxygen was of the same order in both techniques, with 12 per cent. oxygen the dry weight per nodulated plant in the root-enclosure experiment (1951) was over five times greater than the corresponding value for the whole-plant enclosure method, although the duration of growth and the season of year (April and May) were similar.

TABLE III

Effect of Oxygen Supply on Dry Weight attained by Clover and Soya-bean Plants, Relative Basis, Root-enclosure Method

% oxygen supplied.	Clover.		Soya-bean.*	
	Nodulated.	Non-nodulated.	Nodulated.	Non-nodulated.
21	100	100	100	100
12	74	100†	84	93
5	50	71	64	94
1	9	21	—	—

* The figures for Soya-bean are the means of the values reported by Bond (1951*a*) for separate experiments.

† This figure is an estimate rather than an actual result. See p. 390.

Reduced light intensity inside the aspirator-bottles was probably mainly responsible for the poorer growth. Measurements showed that, owing to reflection, the shading effect of the stopper, and the presence of condensed moisture, there is likely to be at least a 50 per cent. reduction of light within such a bottle as compared with the intensity prevailing in the greenhouse. Any attempt to increase the latter by reduced use of roof-screens resulted in undesirably high temperatures within the bottles. Another possibly detrimental feature is that for much of the time the plants are exposed to a saturated atmosphere within the bottles, which is an unnatural condition for clover. Greater success would doubtless attend the method if it were possible to select another legume of similar habit to clover, but of which seed of greater genetic uniformity was obtainable. Also the question of the best level of carbon dioxide for use in this type of experiment probably requires further investigation.

The dry weights (per plant) of the red clover grown by Wilson and Fred

(1937) by the whole-plant enclosure method are mostly lower than the corresponding values in the present authors' experiments, but after repeated experiment the former authors concluded that a partial pressure of 0·05 atm. oxygen could be singled out as a critical level, below which marked curtailment of growth occurred. In partial agreement with this is the finding that in the root-enclosure experiments now described the greatest reduction of growth attended the 5 per cent. to 1 per cent. interval in diminishing oxygen supply. The reduction was, however, more marked in nodulated than in combined nitrogen plants. Moreover there were clearly marked responses at higher levels of oxygen, again with differences between the two types of plant. The general conclusion is that Wilson and Fred's results were in the right direction but were incomplete.

It is appreciated that the greater usefulness of the root-enclosure method is contingent on an absence of any significant internal diffusion of oxygen, through the plant, from the shoot to the root system. This question was fully discussed with reference to Soya-bean in a previous paper (Bond, 1951*a*), where it was concluded that there was no evidence of such diffusion. The oxygen data obtained in the clover experiments point to the same conclusion. In addition the extreme difference in size of plant between the high and low oxygen treatments (Pl. XX, Fig. 3) leaves no doubt that the amounts of oxygen available to the root systems were in fact very different.

Finally, brief reference will be made to the effect of oxygen tension on nodule development. In the 1951 root-enclosure experiment the mean numbers and dry weights of nodules per plant were as follows:

	Number.	Dry wt. (mg.).
21% oxygen . .	231	26
12% ,, . .	248	29
5% ,, . .	328	20
1% ,, . .	178	3

The differences between means are all significant ($P = 0{\cdot}05$) except for those between 21 and 12 per cent. oxygen. It therefore appears that, of the four levels of oxygen employed, 5 per cent. is most favourable to nodule initiation. That so many nodules should form at 1 per cent. oxygen is surprising, considering the extreme reduction in root growth (shown by the combined nitrogen plants also) and consequently in opportunities for infection. It is inferred that the infective powers of the bacteria are but little affected even by this degree of oxygen limitation. From the dry-weight data it is clear that at 1 per cent. oxygen the nodules remained very small, and also that those at 5 per cent. were on an average of smaller size than at higher oxygen levels. Since it has already been concluded that, of the four tensions of oxygen used, nodules function best at 21 per cent., there appears to be a marked difference between the optimal oxygen levels for nodule initiation and for the later stage in which the nodules function as centres of nitrogen fixation, the oxygen requirement of the latter stage being higher.

SUMMARY

A comparison has been made of the effect of oxygen supply on the growth of (*a*) nodulated red clover plants dependent on nitrogen fixed in the nodules, and (*b*) non-nodulated plants provided with ammonium-nitrogen.

Two techniques have been used. In the first the plants were grown in water culture with the root system only exposed to adjusted oxygen supply. In the second the plants were rooted in sand and were wholly enclosed in large aspirator-bottles, the atmospheres of which were adjusted to different oxygen contents. Four levels of oxygen were employed, namely, 21, 12, 5, and 1 per cent.

With the first method, each step in the reduction of oxygen supply resulted in a significant curtailment of growth in nodulated plants. In comparison the responses of the ammonium-plants lagged behind, leading to the conclusion that the effective functioning of nodules necessitates a higher level of external oxygen than in the case of roots. Given a favourable oxygen supply, plant growth was very vigorous in this method.

The number of nodules was greatest at 5 per cent. oxygen, indicating that in clover the optimum oxygen level for early nodule development is well below that for the subsequent functioning of the nodules as centres of nitrogen fixation.

Under similar greenhouse conditions plants grown by the second method were in general much smaller. No clear effects of variation in oxygen supply were obtained, the reason being, it is concluded, that growth was limited as a result of the total enclosure of the plants, the light factor being particularly implicated. Of the two methods that of root-enclosure is therefore preferred.

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One of us (T. P. F.) held a Carnegie Research Scholarship during part of the period of this investigation. The authors are indebted to Mr. A. C. Crundwell for statistical advice, and to Mr. W. Anderson and Miss K. McCormick for photographical and technical assistance respectively.

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EXPLANATION OF PLATES

Illustrating T. P. Ferguson's and G. Bond's article on 'Symbiosis of Leguminous Plants and Nodule Bacteria. V. The Growth of Red Clover at Different Oxygen Tensions'

PLATE XIX

Figs. 1 and 2. Nodulated plants supplied with 21 and 12 per cent. oxygen respectively, 1953 root-enclosure experiment. (×$\frac{1}{9}$.)

Figs. 3, 4, and 5. Nodulated plants supplied with 12, 5, and 1 per cent. oxygen respectively, 1951 root-enclosure experiment. (×$\frac{1}{9}$.)

Figs. 6, 7, and 8. Combined-nitrogen plants (non-nodulated) supplied with 21, 5, and 1 per cent. oxygen respectively, 1951 root-enclosure experiment. (×$\frac{1}{10}$.)

PLATE XX

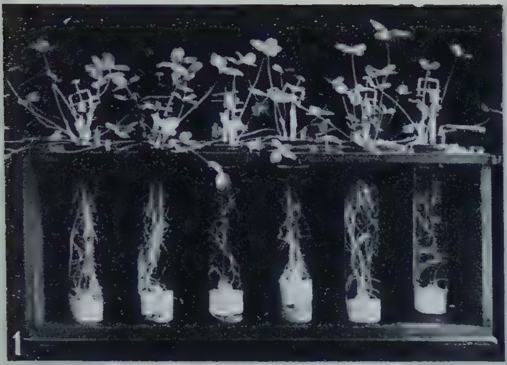
Fig. 1. Part of root system of a nodulated plant, root-enclosure technique, 21 per cent. oxygen. (Nat. size.)

Fig. 2. Single culture-tube from root-enclosure experiment. (×$\frac{1}{2}$.)

Fig. 3. Combined-nitrogen plants (non-nodulated) supplied with 21 per cent. (right) and 1 per cent. oxygen respectively, root-enclosure experiment. (×$\frac{1}{7}$.)

Fig. 4. Aspirator-bottles with nodulated plants from whole-plant enclosure experiment, supplied with (left to right) 21, 12, 5, and 1 per cent. oxygen. (×$\frac{1}{8}$.)

Fig. 5. Aspirator-bottle supplied with 1 per cent. oxygen, showing roots growing on surface of sand. (×$\frac{1}{2}$.)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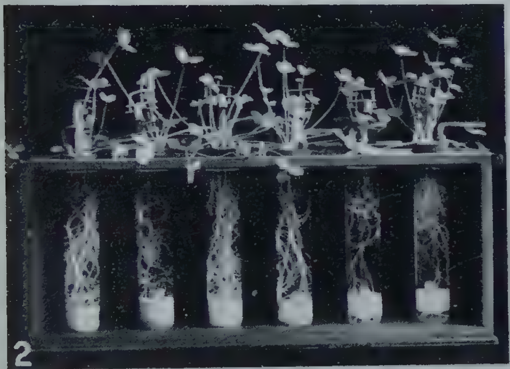
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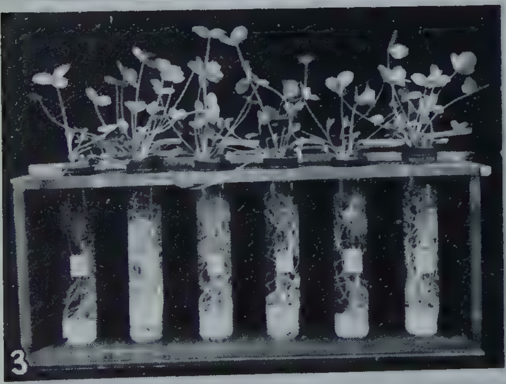
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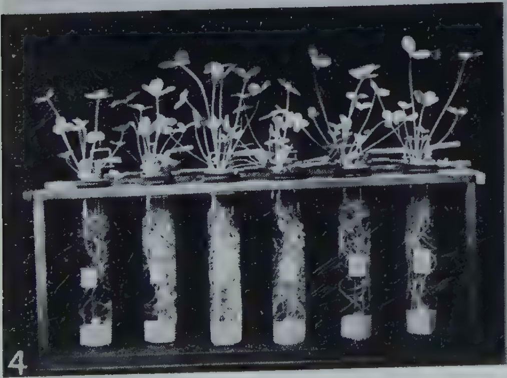
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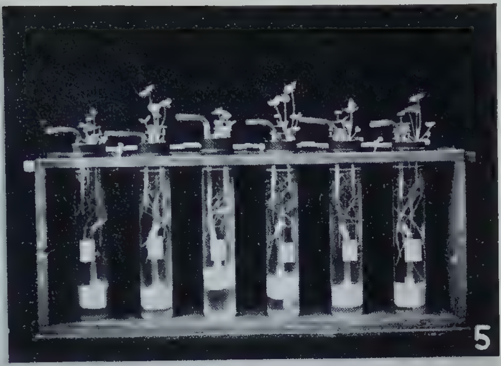
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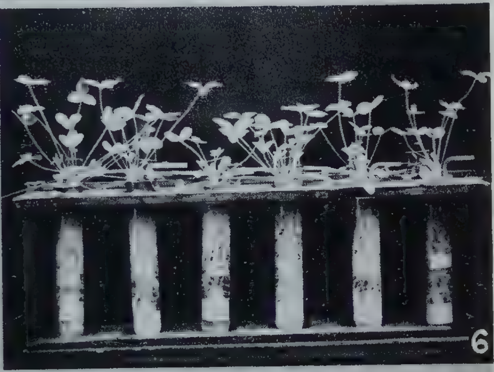
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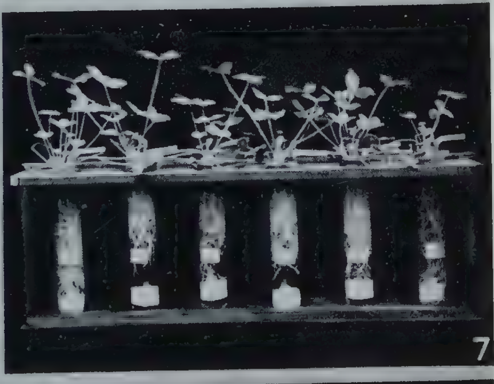
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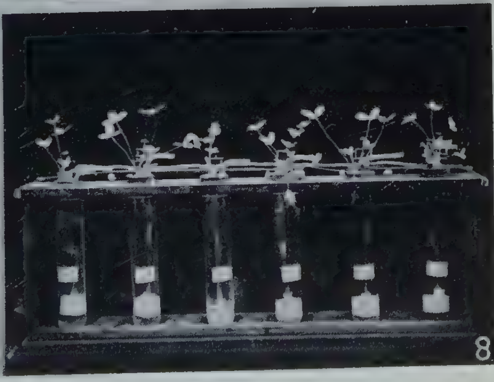
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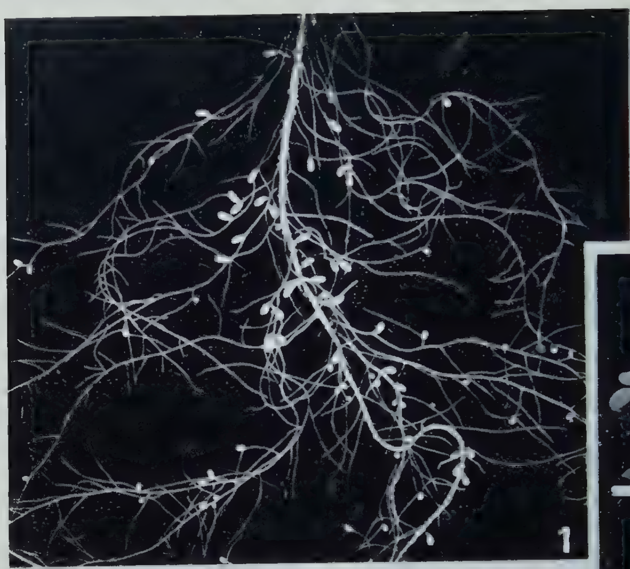
T. P. FERGUSON *and* G. BOND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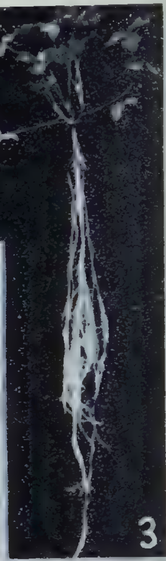
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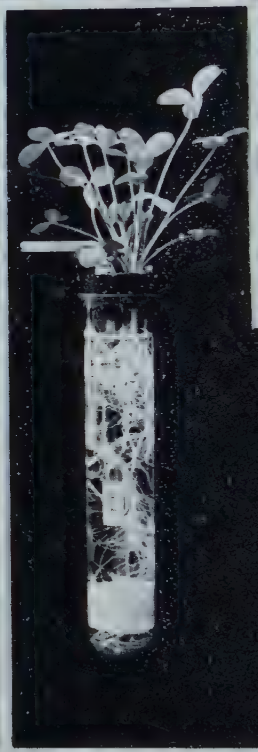
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Experimental and Analytical Studies of Pteridophytes

XXVI. *Ophioglossum vulgatum*: Comparative Morphogenesis in Embryos and Induced Buds

BY

C. W. WARDLAW

(*Department of Cryptogamic Botany, University of Manchester*)

With Plate XXI and ten Figures in the Text

ABSTRACT

The sequence and rate of organ formation in embryos and induced buds of *Ophioglossum vulgatum* are compared. In the embryo, the appearance of the primary root considerably precedes the organization of the shoot apex and the formation of the first leaf; but in induced root and shoot buds the reverse is the case. With the limited nutrition provided by the saprophytic underground prothallus, the first leaf of the embryo (actually the second leaf formed) does not appear above ground until after 7–8 years; but in buds induced in decapitated shoots, four to six new leaves may be formed in 5–6 months. Completely defoliated shoots may show a comparable rate of leaf formation. The conclusion is reached that while the fundamental pattern of development is the same in embryos and buds, the actual sequence in which the primary organs are formed, and their rate of formation and development, are referable to nutritional factors.

INTRODUCTION

WHEN endogenous buds are induced in the roots and shoot of *Ophioglossum vulgatum*, the first evidence of organ formation consists in the differentiation of the shoot apical meristem and first leaf, the formation of the first root usually taking place some time later (Wardlaw, 1953). In the development of the embryo, on the other hand, the foot and a large root are the first organs to be formed, a considerable period of time elapsing before the shoot apex and first leaf can be distinguished (Bruchmann, 1904). The initial patterns of development in embryos and buds thus appear to be rather different, though each culminates eventually in the same adult organization. A scrutiny of the evidence suggests that these differences in developmental pattern are determined by nutritional factors. This hypothesis is examined in the present paper.

THE EMBRYO AND YOUNG SPOROPHYTE

Bruchmann (1904) has given an adequate if incomplete account of the embryogeny in *O. vulgatum*. The zygote divides by transverse and vertical walls to yield an octant stage, and thereafter further growth and cell division

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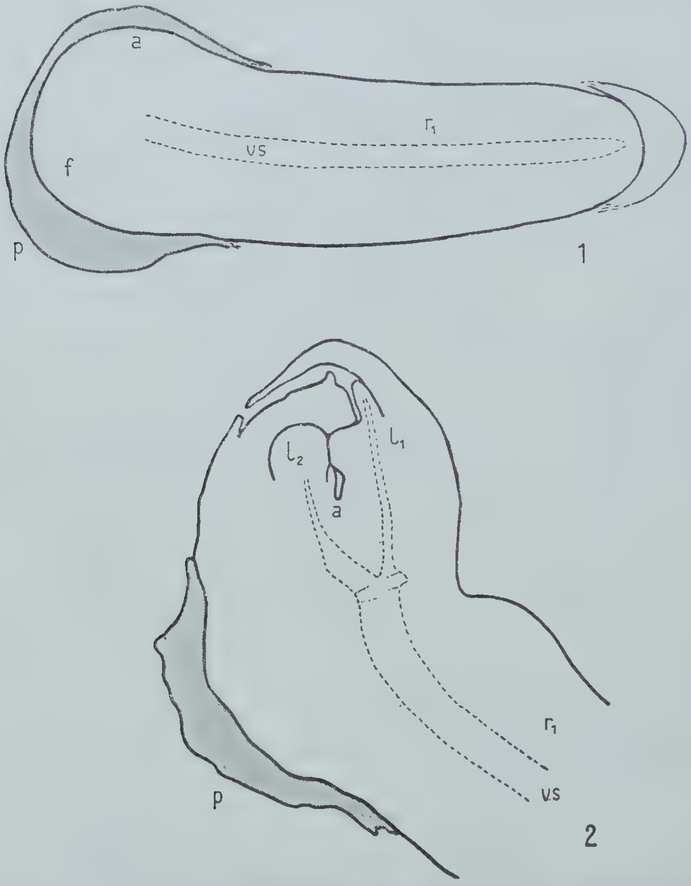
take place, an ellipsoidal body of apparently uniform embryonic tissue being formed. About this stage the initial cell of the first, endogenous, root can be seen in one of the hypobasal quadrants. This quadrant now becomes the seat of active growth, and the conspicuous root which is formed subsequently bursts out of the prothallus (Text-fig. 1). The other hypobasal quadrant develops considerably more slowly into a parenchymatous foot. The vascular strand of the root fades out close to the foot. Meanwhile, the whole of the epibasal region remains in the undifferentiated embryonic state. According to Bruchmann, development to this stage occupies several years. The embryo is stored with nutritive substances, probably mainly carbohydrate, but is still without the endophytic fungus.



TEXT-FIGS. 1, 2. Embryos of *Ophioglossum vulgatum* (redrawn diagrammatically from Bruchmann). Fig. 1. Young embryo, consisting of the primary root (r_1), the foot (f), and the still unorganized apical region (a); p, prothallus; vs, vascular strand. Fig. 2. An older embryo, showing the rudimentary first leaf (l_1), with its leaf sheath enveloping the second leaf (l_2); a, the apical meristem of the shoot is situated at the base of a small channel.

The shoot apex, the first leaf, and a surrounding leaf sheath next become organized in a superficial, approximately median position in the epibasal segment, the leaf being continuous with the first root. The leaf-trace typically becomes conjoined with the root stele. The initials of a second root become visible about this stage, near the base of the first root. The first leaf undergoes some further growth, but it remains small and rudimentary. The second leaf originates at the shoot apex as in the adult plant (Text-fig. 2); its vascular strand also becomes conjoined with the root stele. No vascular tissue can be discerned immediately below the shoot apex. As in the adult plant, stipular sheaths are associated with the first and second leaves. In the course of some 8–10 years the second leaf eventually appears above ground. It is of small size and bears no fertile spike; but the third leaf, which appears above ground the following year, may be fertile. The ensuing developments are as in the adult plant.

Bruchmann has illustrated a number of young sporophytes, either free in the soil or still attached to the prothallus. The interesting thing about these plantlings is that while they may have one to three, or even four, well-developed, horizontally disposed roots, the shoot still consists of a very minute bud. One illustration shows an adventitious bud near the distal end of a longish root. Like the parent prothalli, these young sporophytes are subterranean.

The development of the embryo may now be considered. When it has become a multicellular ellipsoidal body, consisting of apparently homogeneous embryonic cells, there is evidence that differentiation has already begun, e.g. one of the hypobasal quadrants begins to show rapid relative growth and becomes organized as a root, while some time later the first leaf originates in the corresponding epibasal quadrant. The considerable delay in the growth and morphogenetic activity of the epibasal region suggests that a growth-regulating substance, or the supply of nitrogen-containing metabolites necessary for protein synthesis at the shoot apex, may be the limiting factor (or factors). Although the *sequence* of organ formation in the embryo of *Ophioglossum* is different from that in leptosporangiate ferns, such as *Dryopteris* or *Adiantum*, the relationships of the primary organs are closely comparable.

Presumably owing to the limited nutrition supplied by the saprophytic underground prothallus, the initial growth of the embryo is very slow; but a marked acceleration of growth takes place once a leaf has appeared above ground and become active photosynthetically. As the cells of the prothallus and embryo contain abundant starch, carbohydrate supply is not a limiting factor.

As in other embryogenies, the polarity of the embryo in *O. vulgatum*, with the distinction of apex and base, is determined at a very early stage. Thus, although the histological organization of the shoot apex is greatly delayed, the evidence is consistent with the view that it is determined very early. The first leaf, like the second and all subsequently formed leaves, is dorsiventral with the normal orientation towards the shoot apex. Thus, no matter how sluggish the embryonic development in *O. vulgatum* may be, the characteristic growth pattern, not unlike that of other ferns, is apparently established at an early stage and is maintained during the subsequent development.

ORGANOGENIC DEVELOPMENT OF INDUCED BUDS

Early stages in the formation of buds in the cortex of roots and in the pith of decapitated shoots have already been described and illustrated (Wardlaw, 1953). When a bud is formed at the root-tip, according to Rostowzew (1891), a segment of the root apical meristem gives rise, by further cell division, almost simultaneously to the apical meristem and first leaf of the bud. Leaf growth may be well advanced before the first bud root emerges. Similarly, when a bud originates in the cortex of a mature root, there is first of all a phase of rapid cell-division at a centre of growth, an ellipsoidal or spherical mass of meristematic tissue being thereby formed. Within this mass a shoot meristem first becomes organized (Wardlaw, 1953, Pl. XXVIII, Figs. 10–14). Very soon thereafter the first leaf is formed and during the next few months it may show extensive growth. In some buds the initial of the first root may soon be differentiated; in others it may be somewhat delayed. Once the root-tip is differentiated, root growth is rapid, with the result that a thick root, considerably longer than the first leaf, may be formed in 4–6 months (Wardlaw, 1953, Pl. XXVII, Fig. 6). In endogenous buds, formed in the pith of a decapitated shoot, the course of development is closely comparable (Wardlaw, 1953, Pl. XXX, Figs. 20, 21). The shoot apex is first formed, but the first leaf may be differentiated almost simultaneously and thereafter grows rapidly; the root apex is differentiated somewhat later, but its growth may be very vigorous in some instances. In buds formed laterally on the shoot, a shoot meristem is first organized; leaf formation follows, and some time later a root apex appears. In these induced buds the relation of roots to the leaf-bases cannot always be precisely specified.

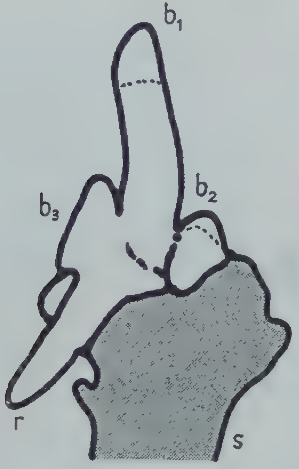
SEQUENCE AND RATE OF ORGAN FORMATION IN INDUCED BUDS

In the stout decapitated shoot illustrated in Text-fig. 3, three endogenous buds were formed at the cut surface in the course of 5–6 months. Two of these, indicated as b_1 and b_2, originated in the pith, while the third, b_3, was formed in the cortex in the usual position of a lateral bud, i.e. in the region of conjunction of two of the shoot meristeles (Wardlaw, 1953). The numbers of leaves and roots in these three buds were as follows:

	Leaves	Roots
Bud 1	1	1
,, 2	4	1
,, 3	3	3

As shown diagrammatically in Text-fig. 4, the first root of *bud 3* is on the opposite side of the shoot to *leaf 1*; but *roots 2* and *3* occupy approximately basal positions to *leaves 2* and *3* respectively. These massive roots are a conspicuous feature. *Leaves 1* and *2* are not quite diametrically opposite each other; *leaf 3* lies between them as indicated. The bud stele is solenostelic and is formed entirely from decurrent leaf-traces which have widened tangentially.

Organ development in *bud 2* is illustrated diagrammatically in Text-fig. 5, and the downward differentiation of its vascular tissue in Pl. XXI, Figs. 2–6. In this bud, the smallest of the three, the first leaf (l_1) is small and rudimentary and can only be seen in sections taken near the base of the bud. The conspicuously developed leaf is *leaf 2*. Two leaves in an actively growing condition are present near the shoot apex (Pl. XXI, Fig. 1). The single root (r_1), which can be seen about the same level as *leaf 1*, is basal and lateral to *leaf 2*. The bud stele, which is small and circular to elliptical in transverse section about the level of *leaf 1*, can be traced downwards into the pith of the parent shoot where it gradually fades out (Pl. XXI, Figs. 2–6). This material provides evidence for the view that the vascular tissue owes its inception to the basipetal diffusion of substances from shoot and/or leaf apices. Below the base of *leaf 1* the differentiating vascular tissue gradually spreads out, so that an increasing number of the cells of the pith parenchyma is affected. They are, in fact, stimulated to active division (Pl. XXI, Figs. 3, 4). This increasing number of dividing cells is just what would be expected to follow the downward diffusion of a stimulatory substance. Lower down, the division of individual cells is less intensive but the number of pith cells in which some division has been induced is greatly increased (Pl. XXI, Figs. 5, 6). Scattered



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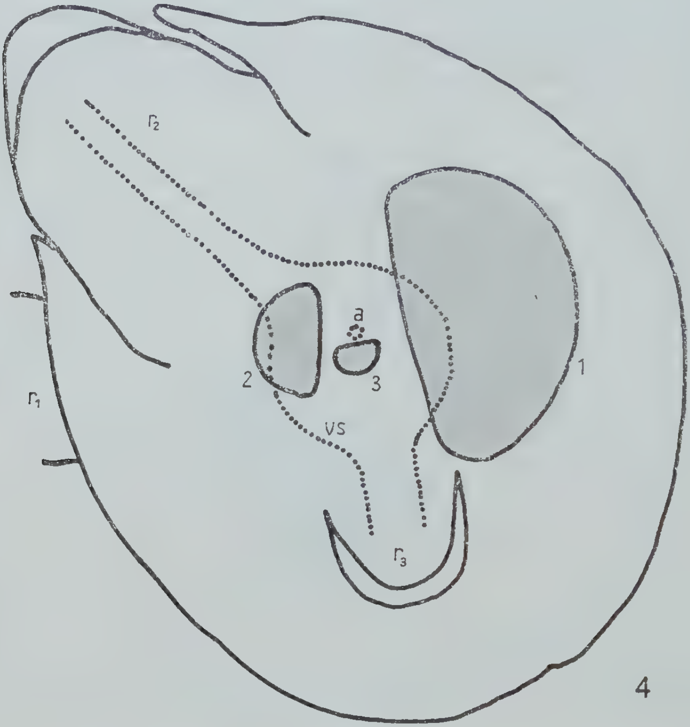
TEXT-FIG. 3. Three buds (b_1–b_3) induced in the pith and cortex of a decapitated shoot (s); r, root. (×5.)

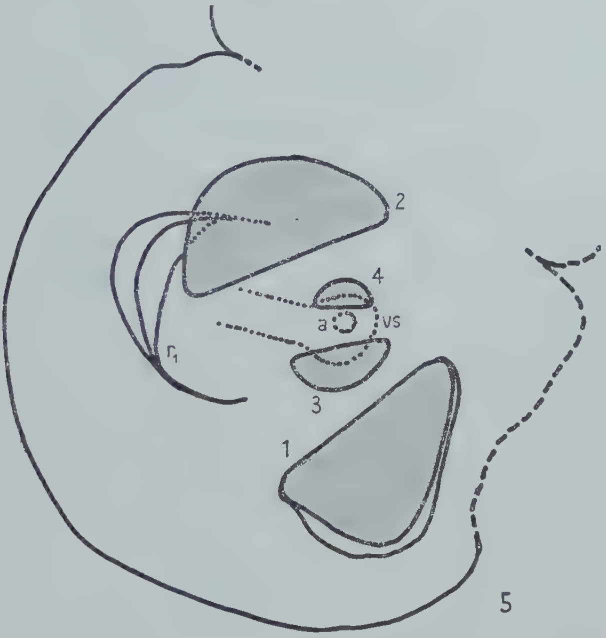
through the pith at these lower levels, occasional lignified tracheidal, or pseudo-tracheidal, cells can be observed (Pl. XXI, Fig. 6). An evident effect of the substance responsible for the inception of vascular tissue is that parenchymatous cells are induced to divide actively so that eventually a mass of quite small meristematic cells is present within the original cell envelope.

The largest bud, indicated in Text-fig. 3 as b_1, was peculiar in that it consisted of a single large leaf, enclosed in its sheath, and a single root which penetrated downwards into the cortex of the parent shoot. A shoot apex could not be distinguished in the serial cross-sections. This single leaf, however, is not centric: its distal region is typically flattened out into a lamina as in the normal leaf development in this species, and its vascular anatomy is normal.



TEXT-FIG. 4. Composite diagram, prepared by superimposing camera lucida drawings, showing the positions of the leaves and roots of *bud 3* in Text-fig. 3 as seen in transverse section. 1, 2, 3, and r_1, r_2, r_3, leaves and roots respectively, in the order of their formation; *a*, position of shoot apex; *vs*, vascular system of shoot. (×55.)

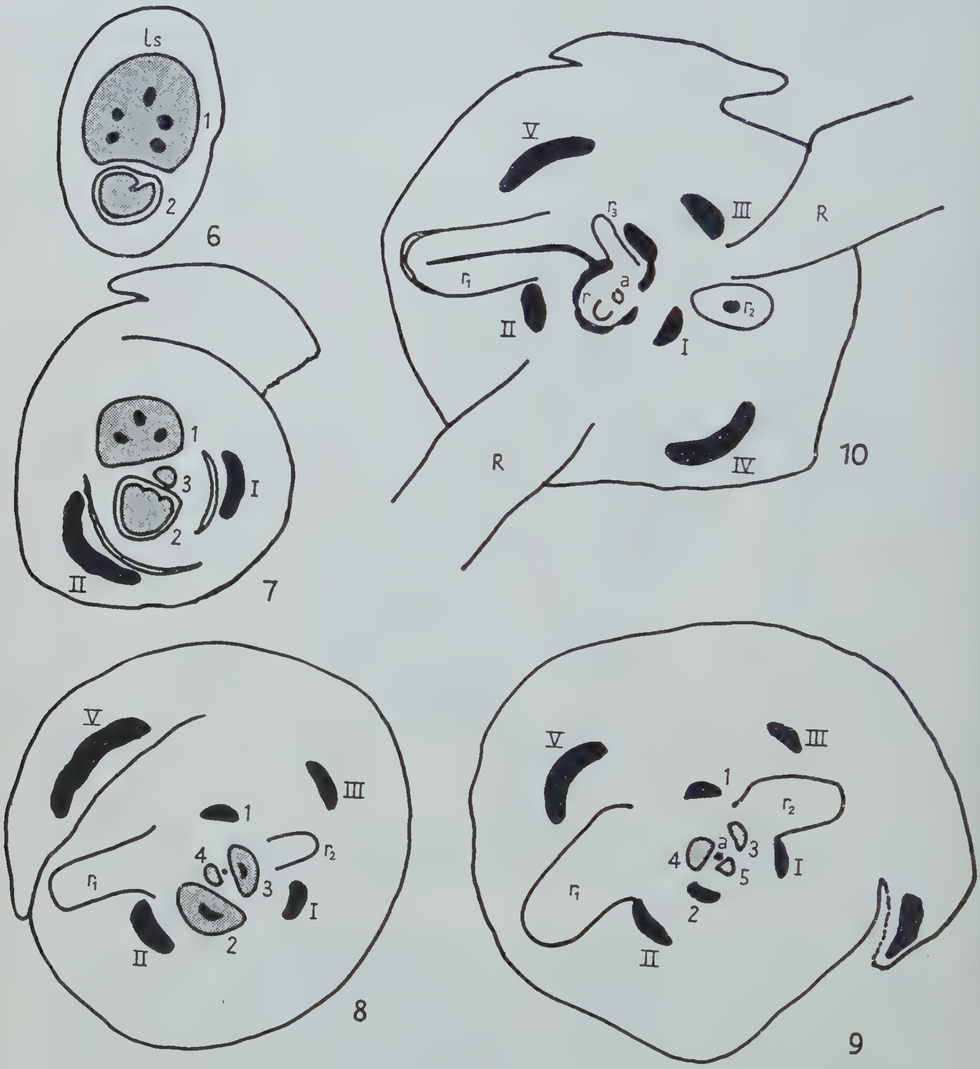
In another decapitated shoot a small, almost callus-like growth but, in fact, a small bud, developed from the pith. This bud had four leaf premordia and two roots, the latter being lateral and basal to the first two leaves. *Leaf 1* had the characteristic limited growth of a prophyll: its distal region had soon become parenchymatous and its vascular strand had faded out acropetally. The vascular system of the axis was formed by the decurrent leaf traces which near the base of the bud formed an incompletely differentiated solenostele. Below this level the vascular tissue faded out basipetally in an irregular manner, the strands of the first two leaves persisting and becoming conjoined each with a root stele. There was little evidence of vascular continuity with the stele of the parental shoot.



TEXT-FIG. 5. Composite diagram of *bud 2* in Text-fig. 3; four leaves and one root are present. Lettering as in Text-fig. 4. (×55.)

LEAF FORMATION IN DEFOLIATED SHOOTS

Large, strongly growing plants were defoliated leaf by leaf till eventually the apical meristem was exposed. In some specimens the last formed, and still very young, leaf primordium may not have been excised. These plants were placed with the apex at the soil surface in pots and kept in a cool greenhouse. In the course of 6 months (June–December 1951) a considerable amount of growth had taken place and a pale green cone surmounted the end of the shoot. The following year a small green leaf was expanded. When one of these plants was fixed and sectioned after 6 months' growth, it was found that it had formed five new leaves and leaf primordia in normal phyllotactic sequence with those originally present, and four new roots (Text-figs. 6–10). In another, four new leaves but only one root had been formed. In yet another specimen, six new leaves (including one very young primordium) had been formed, i.e. primordium had been formed at approxi-



TEXT-FIGS. 6–10. Serial sections in basipetal sequence showing leaf and root formation in a completely defoliated shoot. 1, 2, 3, &c., new leaves in the order of their formation; I, II, III, &c., original leaves of the shoot, in the order of their increasing age; *R*, roots originally present; r_1, r_2, &c., new roots in the order of their formation; *ls*, leaf sheath; *a*, position of shoot apex. Vascular tissue is indicated in solid black. (× 12.)

mately one per month. The new roots were formed in a close sequence round the apex; in one plant, two of them, which had their inception close to the apical meristem, penetrated downwards into the pith. In the matter of leaf formation, these defoliated shoots, as compared with embryos and normal plants, are very active indeed. In this they resemble induced buds.

THE MATURE PLANT

In a well-grown plant of *O. vulgatum* six leaves may be present, these including the expanded leaf of the current year, four leaves at different stages of development (the oldest of which will be the foliage leaf of the following year), and a recently formed primordium at the shoot apical meristem. Smaller plants may have five or four leaves. One leaf usually appears above ground each year, and growth and organogenesis apparently proceed in a slow but regulated fashion year after year. It may be inferred that one, and occasionally two, new primordia are formed each year. In decapitated shoots, on the other hand, buds induced in the pith may form two leaves in the course of 3–5 weeks and after 5–6 months some four to six new leaves may be formed. In about the same period, buds formed near the root-tip or in mature regions had two to three leaves. These records show that, during the period immediately following the organization of a bud meristem, new leaves are formed in relatively rapid sequence. But after some four to six leaves have been formed, by which time the older ones have become massive, and the oldest one petiolate and laminate, the process of leaf formation at the apex apparently slows down and becomes regulated so that only one or perhaps occasionally two leaves are formed each year. These facts are confirmed by the results obtained in defoliation experiments. Whether the slowing down in the rate of formation of new primordia is determined by the nutritional requirements of the older but still actively growing leaves, or whether it is due to some substance produced during the photosynthetic activity of the annual leaf, is not known. In *O. vulgatum*, as in other ferns and flowering plants, the formation and growth of the younger leaves appear to be regulated by older ones, but the precise nature of the mechanism has still to be ascertained.

DISCUSSION

This inquiry into the sequence and rate of organ formation and development in embryos, buds, and defoliated shoots of *Ophioglossum vulgatum* has thus brought to light some notable differences. In embryos, root formation considerably precedes the organization of the shoot; in induced buds the reverse is the case. Nevertheless the fundamental relationship, or the underlying pattern of development, of the shoot meristem, the first leaf and the first root, appears to be closely comparable in embryos and buds. The differences indicated above are probably attributable to differences in the nutrient-supplying power of the subtending tissues, i.e. those of the gametophyte and sporophyte respectively. This suggestion is borne out by other facts of the embryonic development. Thus, the first green leaf of the embryo, actually the second leaf, may take 8–10 years to appear above ground, whereas in induced buds a new leaf may be formed and appear above ground in the course of a year.

Among the materials supplied to embryos and buds by their subtending tissues, growth-regulating substances are probably important. The young embryo is apparently sufficiently well supplied with the auxin and other substances essential for root formation and growth, but it is insufficiently supplied with the substances necessary for the full organization and active growth of its shoot apical meristem. In induced buds, by contrast, the apical meristem is apparently well supplied with the necessary substances, for it is a region of rapid metabolism and morphogenetic activity. Root and shoot buds are characterized from the outset by the formation of densely protoplasmic cells, whereas young embryos are characterized by starch-filled cells. In short, the difference in the sequence of organ formation in buds and embryos appears to be largely, if not entirely, referable to the nutritional resources of the subtending tissues. Buds derive their nutrients from a sporophyte plant with photosynthetic leaves and a well-developed root system, whereas embryos draw upon an underground saprophytic prothallus.

ACKNOWLEDGEMENT

The author has pleasure in thanking Mr. E. Ashby for his help in microscope preparations and photographic illustrations.

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EXPLANATION OF PLATE

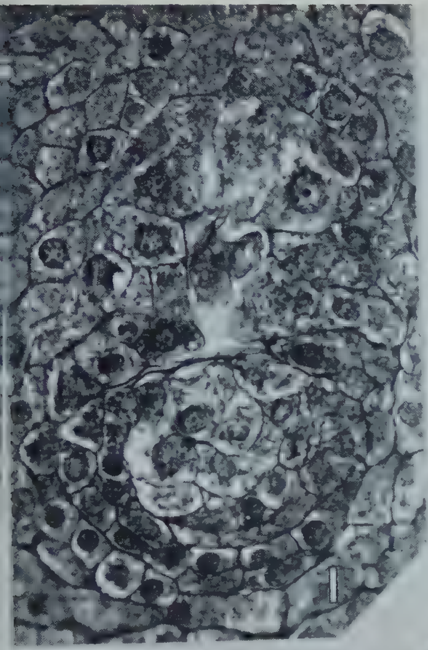
Illustrating Professor C. W. Wardlaw's paper on 'Experimental and Analytical Studies of Pteridophytes.' XXVI. *Ophioglossum vulgatum*: Comparative Morphogenesis in Embryos and Induced Buds.' (All figures are from untouched photographs; × 225.)

Figs. 1–6. A basipetal series of transverse sections of *bud 2*, illustrated in Text-figs. 3 and 5, in which four leaves and one root were present.

Fig. 1. This section is taken just above the shoot apical meristem and shows primordial leaves 3 and 4.

Fig. 2. The small differentiating vascular system some distance below the apical meristem.

Figs. 3–6. These sections illustrate the basipetal differentiation of incipient vascular tissue from cells of the pith parenchyma: in Fig. 3 the pith cells have been stimulated to very active division; in Figs. 4–6 cell division is progressively less intensive but a wider area is affected. A lignified tracheide can be seen near the centre of Fig. 6.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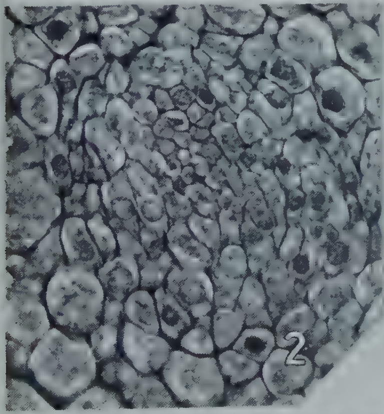
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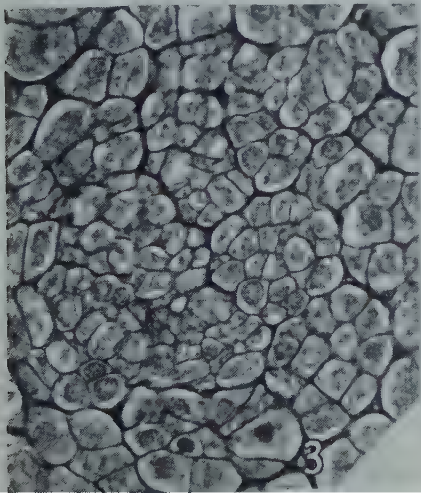
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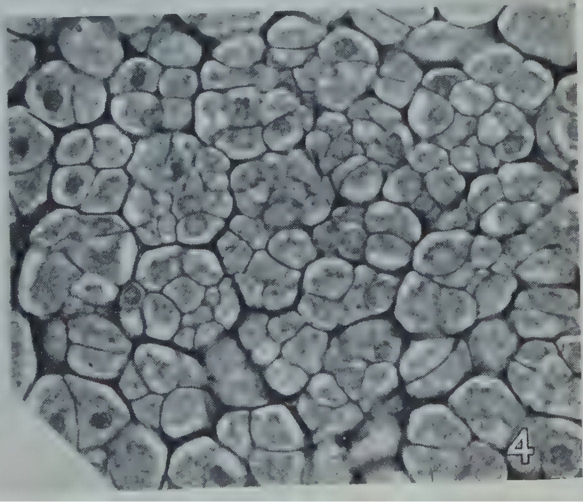
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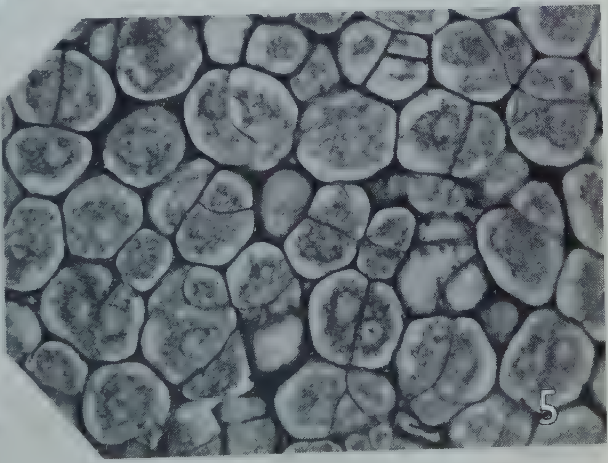
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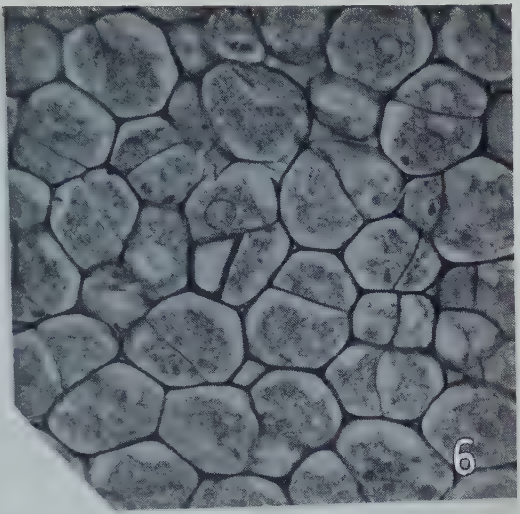
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The Morphology of a New Species of Pteridosperm Seed from the Yorkshire Coal Measures

BY

H. S. HOLDEN

With Plate XXII and seven Figures in the Text

ABSTRACT

The paper gives an account of the morphology of a hitherto undescribed pteridosperm seed the specimens of which come from the Yorkshire Coal Measures and for which the name *Physostoma stellatum* is suggested. Structurally it resembles *Physostoma elegans* in possessing an integument which, apically, consists of a number of separate segments and in the fact that the apex of the megaspore and its associated nucellar tissues bulge into the base of the pollen chamber. It differs from that species in a number of other characters which justify separate specific rank.

SUBSEQUENT to the pioneer work of Williamson (1877) our knowledge of the morphology of European Coal Measure pteridosperm seeds is based in the main on the investigations of Scott (1904, 1923), Oliver (1904, 1909, 1911), Salisbury (1911, 1914), and their co-workers. Seeds of some of these, notably the genera *Lagenostoma*, *Physostoma*, and *Trigonocarpus*, are not uncommon, but in 1911 Oliver and Salisbury (1911) gave a detailed description of two species of *Conostoma*, a genus established for a rare type of seed by Williamson in 1877. The specimens of one of these, *C. oblongum*, were found over a period of years in nodules from the Lancashire and Yorkshire Coal Measures, but those of a second and new species, *C. anglo-germanicum*, had mostly been collected by Dr. Jongmans of Leiden from the Duisberg coal-field in Rhenish Prussia. In addition to the Jongmans material four specimens of *C. anglo-germanicum* from Langendreer, Westphalia, and three from the Lancashire Coal Measures were also available for study. Included among the seed sections examined by Oliver and Salisbury was an obliquely transverse one (R113) which the authors provisionally, and with considerable reserve, assigned to *C. oblongum*. The fortunate discovery of additional specimens of this seed demonstrates that it belongs to a hitherto undescribed species the morphology of which is the subject of the present study. The additional specimens consist of a somewhat indifferently preserved transverse section in the collection of the Geological Survey and Museum and a series of thirty-one slides of a slightly crushed and distorted seed prepared by the peel method elaborated by Walton (1951) and others (Barnes, Duerden, 1931). The section, R113, which is in the University College, London, Collection, was obtained from a Halifax nodule, the Geological Survey specimen came from a nodule

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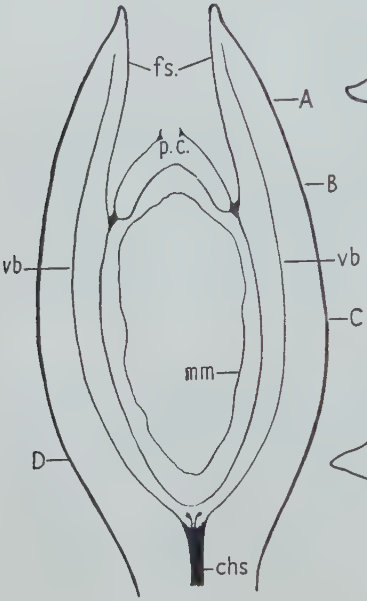
collected at Fieldhouse near Huddersfield, and the serial sections from one collected at Deighton. The specimens are thus all from localities in Yorkshire.

DESCRIPTION OF THE SEED

The seed is a small ovoid one with a maximum diameter of about 1·25 mm. and an estimated length of 2·75 mm. It is radially symmetrical and, in common with that of other pteridosperms, possesses a single integument. This, in its apical part, consists of a ring of separate segments or tentacles six or seven in number. The tips of these segments as seen in transverse section are rounded but, at a slightly lower level, are shaped like equilateral triangles with rounded corners and with the bases of the triangles forming a discontinuous sheath round the apex of the pollen chamber (Text-fig. 2 and Pl. XXII, Fig. 1). The separate segments soon coalesce to form a continuous structure which is strongly ridged externally, the ridges remaining prominent throughout the length of the seed and giving it a markedly stellate outline as seen in transverse



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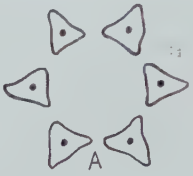

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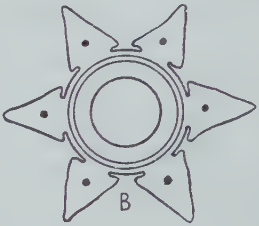

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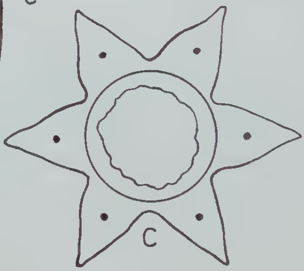

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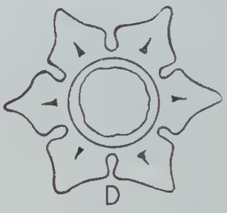

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TEXT-FIG. 1. Diagrammatic median vertical section of the seed. *fs*, free segments (tentacles) of integument; *vb*, integumental vascular strands; *chs*, common chlazal vascular strand; *pc*, pollen chamber; *mm*, megaspore membrane.

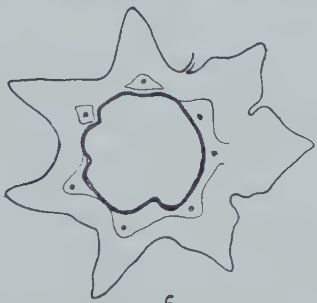
TEXT-FIG. 2. Transverse section of seed near apex at level indicated by 'A' in Text-fig. 1.

TEXT-FIG. 3. Transverse section of seed at the level of the pollen chamber and passing through the apical nucellar bulge—level 'B' of Text-fig. 1.

TEXT-FIG. 4. Transverse section approximately through middle of seed—level 'C' of Text-fig. 1.

TEXT-FIG. 5. Transverse section near base of seed at level 'D' of Text-fig. 1.

section. At first the segments are linked together by a thin ring of integumental tissue and each shows a basal constriction (Text-fig. 3 and Pl. XXII, Fig. 3), but the area of attachment to the underlying part of the integument broadens as the middle of the seed is approached and the basal constrictions disappear (Text-fig. 4 and Pl. XXII, Figs. 4, 7). As the diameter of the seed narrows towards its base the shape of the ridges again changes the area of attachment of each ridge to the underlying tissues, becoming narrowed with the individual ridges being separated from one another by U-shaped grooves (Text-figs. 5 and 6; Pl. XXII, Figs. 6, 8). In contrast to the condition found in *Conostoma* and *Lagenostoma* and in common with that characterizing *Physostoma elegans* to which it seems clearly related, the tissues are essentially parenchymatous. There is no tissue comparable with the resistant, thick-walled sclerotesta which occurs in the other two genera, although towards the base of the seed the cell-walls are thicker than those nearer the apex. This greater thickening is very clearly shown in R113, but it is highly probable that we are dealing in this case with a more mature seed since the megaspore membrane is also much thicker in this specimen than in the other two (Pl. XXII, Fig. 8).



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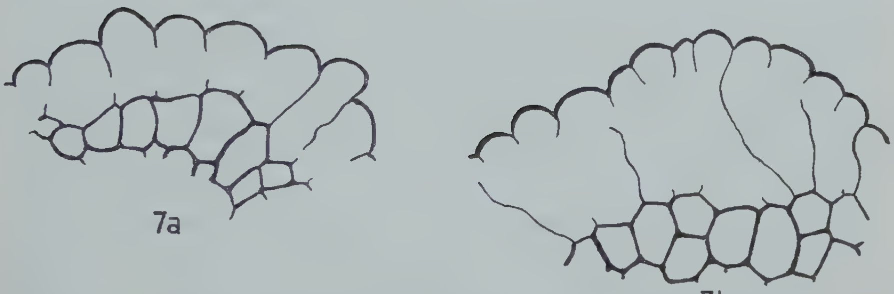

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TEXT-FIG. 6. Camera-lucida tracing of type specimen showing the change in the outline of the integumental ridges, the position of the vascular bundles, and the thick megaspore membrane.

TEXT-FIG. 7*a*. An early stage in the breakdown of the epidermal cells of the seed. TEXT-FIG. 7*b*. A later stage in this process. (× 300.)

The whole of the epidermis constitutes what has been termed by Oliver and Salisbury (1911) a 'blow off' layer extending all round the separate individual tentacles (Pl. XXII, Fig. 1) and covering the whole of the external surface of the integument subsequent to their fusion. The 'blow off' layer is regarded by these authors as consisting of mucilage-containing cells and, indeed, in one of their figures of *Conostoma oblongum* (Pl. 11, Fig. 13*pc.*) the actual cones of mucilage causing the partial disruption of the epidermal cells are quite clearly shown. Similar cones of mucilage are also illustrated in the epidermis of *Lagenostoma Lomaxi* by Oliver and Scott (1904, Pl. V, Fig. 12). The mucilage-secreting areas of the integumental epidermis in both *Conostoma* and *Lagenostoma* are, however, apparently localized. In the seed now described every stage is shown from intact cells to others in which almost complete disruption of the epidermal cells has occurred. An early stage in this process of disruption is shown in Text-fig. 7*a* and a more advanced stage in Text-fig. 7*b*: the same features are illustrated in Pl. XXII, Figs. 1–4. A comparable breakdown of the mucilage-secreting superficial cells of the testa is shown by the seeds of many existing plants, of which *Linum*, *Sinapis*, *Lepidium*, *Plantago Psyllium*, and *P. ovata* are familiar examples. These cells have been the subject of investigation by a number of workers, of whom Tschirch (1889), Wiesner (1928), and, more recently, Skyrme (1935) may be mentioned. Tschirch's figure of *Sinapis alba* shows a condition not unlike that characterizing the early stages of epidermal cell-breakdown in the fossil seed. Immediately below the epidermis is a layer of cortical tissue two or three cells wide, the individual cells of which have slightly thickened walls. Internal to this layer is a zone of smaller, thinner-walled cells interspersed with isolated cells having thick, dark walls. These frequently have dark contents and may have been secretory in character. The vascular supply consists of a series of unbranched strands equal in number to that of the tentacles and the corresponding prominent integumental ridges. They arise presumably from a common central bundle in the chalaza, each strand lying in the centre of the tissue forming the ridge and being continued upwards into the associated tentacle. In some instances the site of the vascular bundle is represented by a cavity or lacuna which appears to be due to the collapse and decay of the constituent tissues, but where destruction is less complete the centre of the lacuna is occupied by a small group of from four to six scalariform or spirally thickened xylem elements. Occasionally traces of a very delicate parenchymatous tissue composed of cells of small calibre are found surrounding the xylem and extending outwards to the more robust tissue of which the main part of the integument consists.

In their description of the University College section (R113) Oliver and Salisbury describe the lacunae as secretory passages but, as indicated in the camera lucida sketch (Text-fig. 6), a vascular bundle consisting of a group of tracheides lies in the centre of five of the seven 'passages'. In the other two the tracheidal strand has moved inwards towards the outer boundary of the nucellus. In the Text-fig. 1 of the paper by these authors the position of this section is plotted, obviously very doubtfully since it is accompanied by a question mark, as being through the chalazal end of the seed, but the fact that the plane of section is one which cuts through the megaspore itself rules out this interpretation. A comparison of Oliver and Salisbury's figure (Pl. 1, Fig. 9) with Pl. XXII, Figs. 5 and 6, of the present paper and with Text-figs. 4 and 5 demonstrates beyond reasonable doubt that not only is it a section through a seed identical with that now described but that the plane of section passes from about the middle of the seed obliquely towards the base.

With the exception of this section the inner tissues of the integument are so crushed and compressed that little of their structure can be determined, and the nucellar tissues show the same poor state of preservation. In R113, however, on that side of the section cut nearer the base of the seed the close-knit, thicker-walled tissue forming the greater part of the integumental ridge in this region adjoins, on its inner side, a thin band of comparatively loose-textured parenchyma (Pl. XXII, Fig. 8). This looser parenchyma does not appear to have extended upwards for any distance from the basal region of the seed into the parts nearer the apex. On its inner margin it adjoins the layer of flattened nucellar cells surrounding the megaspore.

The pollen chamber, occupying the apical part of the seed, closely resembles that of *Physostoma elegans*. As in that seed, it has the form of a bell-shaped crevice, this shape being due to the bulging of the apex of the megaspore and the adjacent nucellar tissue into the pollen-chamber cavity. The pollen chamber shows no indication of a terminal beak and appears to communicate with the exterior by a small circular pore. A few globular objects which may be pollen grains and one of which shows a triradiate mark occur associated with the lagenostome, but no internal structure can be detected in these. The nature of the outer wall of the pollen chamber is one which calls for some comment. In other related seeds, *Physostoma elegans*, *Lagenostoma Lomaxi*, and *L. ovoides* for example, it is generally agreed that this outer wall is one cell in thickness and, in *L. Lomaxi* at least, has been produced by the separation and modification of the apical portion of the nucellar epidermis. The available evidence, as far as the seed now being described is concerned, indicates that the outer wall of the lagenostome consists of three layers of cells, the outermost layer of which has the form of a hollow truncated cone. This is composed of thick-walled cells forming a sheath which extends downwards to the point at which the individual tentacles unite. Although the state of preservation leaves much to be desired, this sheath can be readily seen in both Fig. 2 and Fig. 4 of Pl. XXII. Internal to this layer there is one of parenchyma composed of larger cells, and internal to this again is a zone of smaller thicker-walled elements. This resistant zone is reminiscent of the condition occurring in *Lagenostoma ovoides* (Prankerd, 1912). It is to be hoped that further specimens in which the apical tissues of the seed are better preserved may be discovered which will either confirm this interpretation or provide evidence suggesting possible alternatives.

DISCUSSION

In their paper on the seeds of the *Conostoma* group Oliver and Salisbury (1911) propose the grouping together of those Palaeozoic seeds which share the following characters: (1) a nucellus and integument confluent up to the level of the 'plinth' (i.e. the tapering free end of the nucellus); (2) a free apical part of the integument consisting of separate lobes or 'tentacles' or a series of fused lobes forming a 'canopy'; (3) a vascular supply consisting of a system of strands traversing the integument longitudinally and passing apically into the individual lobes or their fused representatives; (4) an integumental epidermis which was mucilaginous at least locally; (5) a terminal more or less pear-shaped receptacle, the lagenostome, for the reception of the pollen.

The seeds possessing these characters they divide into three series, namely: (*a*) the Physostomeae typified by *Physostoma elegans*; (*b*) the Conostomeae typified by *Conostoma oblongum* and *C. anglo-germanicum* but also including *Gnetopsis elliptica*; and (*c*) the Lagenostomeae typified by *Lagenostoma ovoides* and *L. Lomaxi*. Seward (1917) in his chapter on Palaeozoic gymnosperm seeds includes all the genera listed by these authors, together with *Sphaerostoma*, in one group for which he has suggested the name Lagenostomales, and this grouping has found general acceptance among palaeobotanists (e.g. Gordon, 1941; Walton, 1953). In the same chapter Seward also indicated that, in his view, the further subdivision of the Lagenostomales was, at that time, somewhat premature. Since 1917 the discovery of hitherto undescribed fossil seeds among the plant material in American coal balls and their study by various investigators has occurred (Arnold, 1938, 1948; Krick, 1932; Neely, 1951; Reed, 1939). In addition there has been the discovery and description by Gordon (1910, 1941) of the complex Lower Carboniferous seed *Salpingostoma dazu* and that of *Calathospermum*, also from the Lower Carboniferous, by Walton (1949). It seems probable that these and further possible discoveries will lead to some modification of the diagnostic characters relied on by Oliver and Salisbury and, ultimately, to the establishment of further groups, but, in the meantime, the conservative attitude adopted by Seward has much to recommend it.

There can be no real doubt, however, as indicated earlier, that the seed which forms the subject of this paper is closely allied to *Physostoma elegans*. Oliver and Salisbury (1911) define the Physostomeae as follows: 'Free parts of the ribbed integument consisting of separate segments surrounding and overlapping the large globose lagenostome which opened by a small orifice inserted on the apex of a low papilla. Plinth rudimentary. The apex of the megaspore cavity projected into the floor of the lagenostome. Ribbing at the base obsolete.' This group definition was founded on *Physostoma elegans*, the only seed in the group of which structural specimens were known, and is, in essentials, that proposed by Oliver in an earlier paper (1909) for the genus *Physostoma* itself. The diagnoses in fact differ only in two minor features, namely that Oliver, defining the genus, describes the nucellus as containing numerous secretory sacs, a diagnostic feature omitted in the later paper, and that in this paper the ribbing at the base of the seed is described as obsolete, a feature not mentioned by Oliver.

Apart from these two features, the structural characters of the new seed agree closely with those indicated by the earlier authors as diagnostic for the genus and it seems logical to assign it to the same genus. Specifically the seed, quite apart from its smaller size, shows marked differences from *Physostoma elegans*. The investment of prominent clavate hairs which characterizes *P. elegans* is replaced by a mucilage-secreting epidermis recalling that of *Conostoma* and *Lagenostoma*. The prominent ribs of the integument are prolonged downwards to the base of the seed and the 'tentacles', six or seven in number, are fewer. No secretory cavities are present in the integument, though scattered cells with dark contents occur which may have a secretory function. It is suggested that the seed should be assigned to the genus *Physostoma* and that a new species *Physostoma stellatum* should be instituted for its reception. If this course is adopted, some modification of the generic diagnosis will be needed and the following is suggested:

PHYSOSTOMA—Williamson

Ovoid radially organized seeds with a single integument fused with the nucellus up to the level of the pollen chamber and extending beyond this to the apex of the seed in the form of separate free segments or tentacles. Vascular supply of integument consisting of unbranched strands equal in number to that of the separate segments, each segment receiving a strand. Integumental bundles arising from a common chalazal bundle. Seed essentially soft-textured with no part of the integument modified to form a sclerotesta. Upper part of the ovule with its associated nucellar tissue projecting into the base of the pollen chamber which, as a consequence, has the form of a bell-shaped crevice.

Physostoma stellatum—Sp. nov.

Seed with the above generic characters possessing an integument with six or seven free apical segments, rounded near their tips but appearing triangular in transverse section at a slightly lower level. Free segments fusing to form a strongly ridged coherent sheath to the ovule, the number of ridges equalling the number of free segments and extending to the base of the ovule. Epidermis of integument consisting throughout of mucilage-secreting cells which undergo partial breakdown. Seed devoid of surface hairs and containing no secretory passages. Approximate dimensions: Length 2·75 mm., broadest diameter 1·25 mm.

Type specimen: An obliquely transverse section in Slide 113 in the University College (London) Collection.

Locality: Halifax.

Other specimens: (1) A transverse section in the Collection of H.M.

Geological Survey and Museum: locality—Fieldhouse, Yorkshire; (2) a series of oblique 'peels' in the author's collection: locality—Deighton, Yorkshire.

ACKNOWLEDGEMENTS

Grateful acknowledgements are due to Professor W. H. Pearsall of University College, London, and to Dr. C. J. Stubblefield, Chief Palaeontologist, H.M. Geological Survey, for facilities for studying the sections in the collections of which they have charge; to Dr. T. E. Wallis of the Pharmaceutical Society for his help in supplying information on the techniques he employs in the examination of seeds possessing mucilaginous seed coats, and to Mr. W. N. Edwards, Keeper of the Department of Geology, British Museum, for his advice in drawing up the diagnosis of the seed. Acknowledgements are also due to the Government Grants Committee of the Royal Society for the loan of a microscope in connexion with this investigation.

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EXPLANATION OF PLATE

Illustrating H. S. Holden's article on 'The Morphology of a New Species of Pteridosperm Seed from the Yorkshire Coal Measures'.

With the exception of Fig. 1 all the photomicrographs illustrating this paper are from negatives by Detective-Superintendent G. Salter, Liaison Officer, Metropolitan Forensic Science Laboratory, New Scotland Yard. None of the negatives or prints have been retouched.

Fig. 1. Section near the apex of the seed showing the separate tentacles. The mucilage-secreting epidermal cells are clearly seen. The irregular structures enclosed by the tentacles may possibly represent sections of hairs. (× 80.)

Fig. 2. Section passing obliquely through the pollen chamber. The bulging of the nucellus into the pollen chamber is evident. (× 40.)

Fig. 3. Oblique section below the level of the pollen chamber showing the linking together of the integumental ridges. (× 40.)

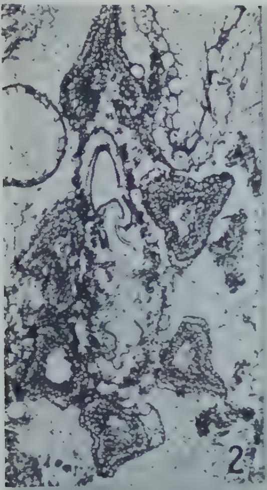
Fig. 4. A neighbouring section. Note the traces of the thick-walled nucellar tissue on the upper right-hand side. (× 55.)

Fig. 5. Section through the middle of the seed. (× 40.)

Fig. 6. Section near the base of the seed. The integumental ridges on lower side show the characteristic change of shape. (× 40.)

Fig. 7. Transverse section of the somewhat ill-preserved seed from the Collection of H.M. Geological Survey and Museum. (× 40.)

Fig. 8. Part of the type specimen (R113) showing traces of the thin-walled tissue between the megaspore membrane and the thick-walled tissue forming the bulk of the integumental ridge. (× 100.)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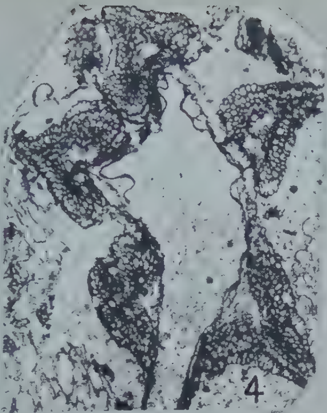
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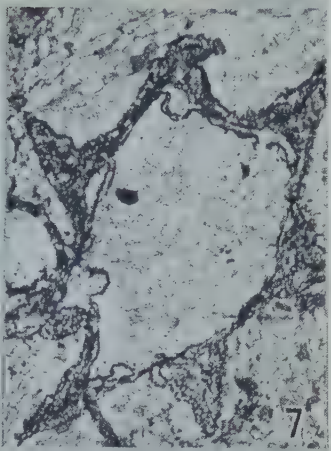
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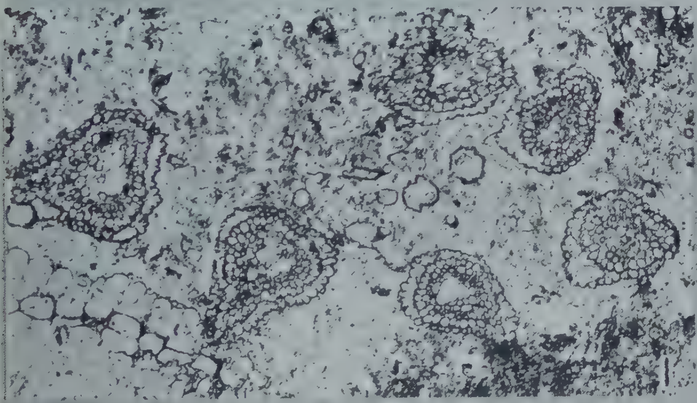
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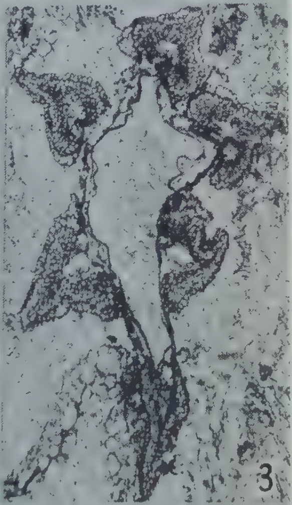
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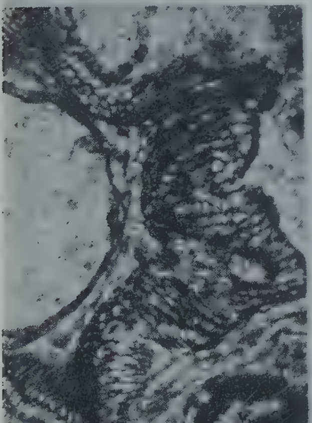
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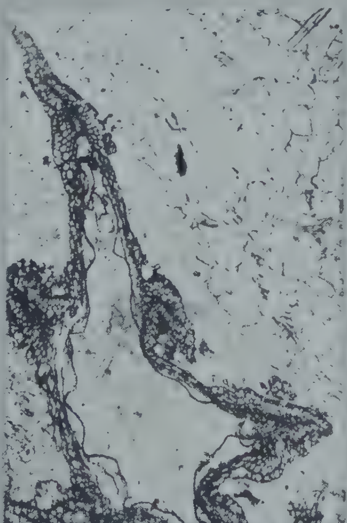
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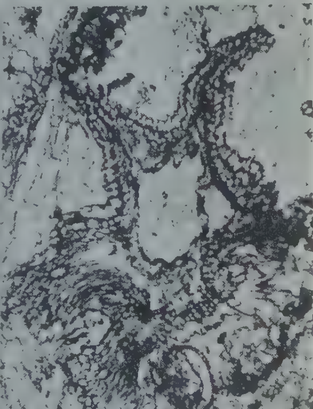
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H. S. HOLDEN

The Nitrogen Metabolism of Groundnut Plants: the Role of γ-methyleneglutamine and γ-methyleneglutamic Acid

BY

L. FOWDEN

(*Department of Botany, University College, Gower St., London, W.C. 1*)

With five Figures in the Text

ABSTRACT

The changes occurring in the nitrogenous compounds during the growth of groundnut seedlings in the dark and light were compared, particular attention being centred on the levels of γ-methyleneglutamine, the principal amide of these plants, and γ-methyleneglutamic acid. The distribution of amino acids and amides in the main organs of normal young and mature plants was also examined. Suggestions are made concerning the possible pathways of synthesis and the functions of γ-methyleneglutamic acid and γ-methyleneglutamine in groundnut plants.

INTRODUCTION

OVER a century before the technique of paper partition chromatography was described for the separation and identification of amino acids (Consden, Gordon, and Martin, 1944), Vauquelin and Robiquet (1806) isolated the amino-acid amide, asparagine, from asparagus juice. Although subsequently a second amino-acid amide, glutamine, was detected in plants by Schulze and Bosshard (1883) and later isolated, no further amides were discovered during the first half of the present century. The idea that the plant kingdom contained only these two amides gained increasing support, and Street (1949) summarized the position in stating that '. . . the complete lack of positive evidence for other amides makes it almost certain that asparagine and glutamine are the only simple acid amides in higher plants'.

Using the techniques of paper and ion exchange chromatography, Done and Fowden (1952) isolated from the vegetative parts of groundnut plants a third amino-acid amide and its corresponding dicarboxylic amino acid, and presented evidence that these substances were probably γ-methyleneglutamine and γ-methyleneglutamic acid. Hereafter γ-methyleneglutamine will be termed γ-MG and γ-methyleneglutamic acid, γ-MGA. Their structures have recently been confirmed by synthesis (unpublished).[1] γ-MG and γ-MGA have also been isolated from tulip bulbs by Zacharius, Pollard, and Steward (1954). A very limited survey of the amino acids occurring in other species made by the present author indicates that the two new compounds are not generally distributed in plants, as are asparagine and glutamine and aspartic acid and glutamic acid.

After further extensive investigation the principal features of asparagine

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[1] Wailes, P., Whiting, M. C., and Fowden, L., 1954: Nature (Lond.), clxxiv. 130.

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and glutamine metabolism in higher plants, described some fifty years ago by Schulze, and by Prianischnikov, remain virtually unchanged (for a comprehensive review of this early work see Chibnall, 1939). The evidence that γ-MG was not present in ungerminated groundnut seeds and that γ-MGA could not be detected in an acid hydrolysate of arachin, the principal reserve protein of groundnut seeds (Done and Fowden, 1952), suggested the possibility that important differences may be encountered between the roles of the new amide in groundnut plants and those of asparagine and glutamine in other species. The investigations to be described in this paper were therefore similar to many of those carried out upon lupin seedlings by nineteenth-century plant physiologists, particularly Schulze, but interest was mainly in the role of the unsaturated amide in groundnut plants rather than that of the saturated amides. The changes in various nitrogenous fractions during groundnut seedling growth in the dark were compared with those occurring in seedlings grown in the light. The distribution of amides and acidic amino acids in the various organs of young and mature plants have also been quantitatively studied, whilst observations have been made upon the amino acids present in developing fruits.

MATERIALS AND METHODS

Growing of seedlings. The groundnut seeds used were from a single large batch of nuts (West African) and were removed from the hulls as required. Preliminary tests had shown that a germination rate of virtually 100 per cent. could be expected. Groups of about fifty seeds were germinated upon moist filter-paper for 3 days at 25°, when the seedlings were planted out into washed sand. They were then maintained in the dark at 25° without the supply of any external nutrients; the sand was kept moist with distilled water. The groups of seedlings were harvested at 2- or 3-day intervals up to the 17th day after the commencement of germination. The stages of development reached during these times were as follows:

3rd day: radicles developed to 2–3 cm. long.
5th day: radicles 5–7 cm. long with slight lateral branching.
7th day: lateral roots developing; cotyledons opening to reveal plumules.
9th day: plumules 2–3 cm. long.
12th day: first two etiolated leaves produced.
14th day: plumules 8–10 cm. long; third leaf developing.
17th day: cotyledons considerably reduced in size; plumules had fourth leaf developing, and roots were much branched.

Another group of seedlings were planted out in sand after 3 days and then maintained in a greenhouse at 24–27° for a further period of 14 days during June under conditions of natural sunlight. When harvested the seedlings were at the three- or four-leaf stage.

Growing of plants. The plants used in the studies of the distribution of the amino acids and amides in different organs were grown normally in soil in a greenhouse during four summer months. The temperature ranged from

23° to 30°. The plants were harvested at 3 weeks, when five leaves had usually developed, and again at 8–10 weeks, when the first flowers were forming and ten to twelve leaves were present on the main stem.

The leaves, together with their petioles, the stems, hypocotyls, and roots of the three-week-old plants, were harvested separately for analysis. From the older plants leaves of different ages were separately analysed, the increasing serial number of the leaves counting from the first well-defined leaf down from the main stem apex being used as an arbitrary measure of leaf age (Pearsall, 1931). Roots from these plants were also analysed. The fruits were allowed to develop on other plants and were analysed qualitatively for the presence of amino acids at two stages of immature development.

Separation of nitrogen fractions

Seventy per cent. ethanol extraction of the fresh plant material was employed to separate the insoluble nitrogen from the soluble nitrogen containing compounds. In the seedling experiments the harvested seedlings were coarsely chopped and then ground to a paste, using a mortar and pestle after the addition of about 50 ml. of absolute alcohol. More ethanol and distilled water were then added in amounts calculated to give a final ethanol concentration of 70 per cent. after allowing for the estimated water content of the fresh plant material. 250 ml. of extraction solvent were thus obtained. The material was extracted with continuous shaking at laboratory temperatures for 24 hours. The insoluble material was sedimented by centrifuging at 2,000 g. for 10 minutes, and the supernatant was decanted and stored in the refrigerator. The residue was twice re-extracted with further 250 ml. portions of 70 per cent. ethanol, each with shaking for 24 hours, and finally washed with 100 ml. of 70 per cent. ethanol. The residue containing the insoluble protein nitrogen was dried at 105° and weighed, whilst the three supernatants and the washings were combined to give the soluble nitrogen containing fraction. A similar extraction procedure was used to fractionate the nitrogen present in the various organs from the older plants, the volume of 70 per cent. ethanol used for each extraction generally being 3 ml. for each gramme of fresh plant material. It was found necessary to concentrate the soluble nitrogen fractions before assaying individual nitrogenous components by removing the greater proportion of the ethanol by distillation under reduced pressure at temperatures less than 40°. Evaporation at higher temperatures may have resulted in appreciable destruction of any glutamine and γ-MG present. The concentrated soluble fractions were finally adjusted to 100 ml.

Estimations of nitrogen fractions

Determinations of the total nitrogen present in the experimental samples were performed following the micro-Kjeldahl digestion procedure of Chibnall, Rees, and Williams (1943). Estimations were carried out in triplicate upon approximately 20 mg. samples of each of the residues and upon a finely ground sample of the ungerminated seeds. Estimates of the nitrogen present in the soluble nitrogen containing fractions were also made in triplicate upon 1 ml. aliquots of each.

The individual nitrogen components in the soluble fractions were estimated as follows:

Ammonia nitrogen was estimated in suitable aliquots chosen to contain between 10 and 60μg. ammonia nitrogen using the Conway unit distillation procedure (Conway, 1939), followed by colorimetric determination by the Nesslerization technique of Umbreit, Burris, and Stauffer (1945).

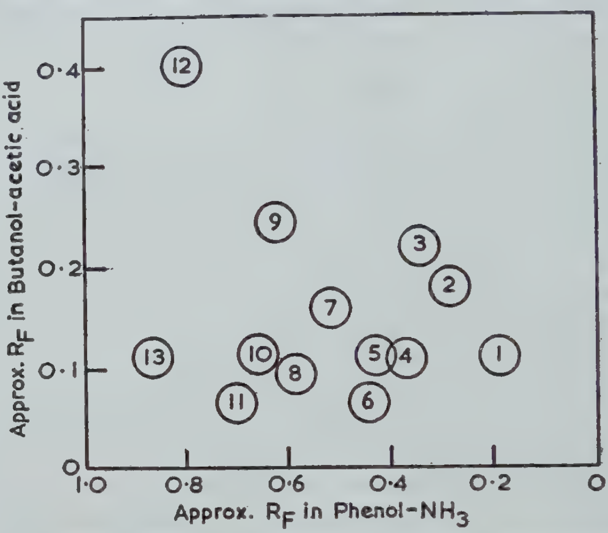


FIG. 1. A diagrammatic representation of a typical chromatogram, showing the positions occupied by the amino acids assayed relative to those of their nearest neighbours. The spots are (1) aspartic acid, (2) glutamic acid, (3) γ-methyleneglutamic acid, (4) serine, (5) glycine, (6) asparagine, (7) threonine, (8) glutamine, (9) alanine, (10) γ-methyleneglutamine, (11) histidine, (12) valine, (13) arginine.

Amide nitrogen was estimated after conversion to ammonia nitrogen by hydrolysis of suitable aliquots in the presence of N . H_2SO_4 for 3 hours at 100°.

Total α-amino nitrogen was determined in aliquots containing between 2 and 10μg. α-amino nitrogen using the colorimetric ninhydrin procedure of Moore and Stein (1948). It was essential to remove ammonia nitrogen also present in the aliquots by the method described by Fowden (1951) before colour development with ninhydrin was commenced.

α-amino nitrogen present in each of the three dicarboxylic acids and their amides was estimated after their separation on two-dimensional paper chromatograms. No difficulties were encountered in completely resolving each of these substances from the other amino acids present in the extracts using the solvent systems described below. A diagrammatic representation of the positions of the spots appearing on a two-dimensional chromatogram is given in Fig. 1.

Five millilitres of each extract were evaporated to dryness *in vacuo* over concentrated H_2SO_4 at laboratory temperatures, and the residues redissolved in suitable smaller measured volumes of distilled water. The volumes were adjusted such that 100μl. of each solution contained between 2 and 10μg. α-amino nitrogen in each amino acid that was to be assayed. Smaller quantities of amino acids could not be accurately estimated, whilst larger amounts tended to be incompletely resolved on chromatograms. Two-dimensional chromatograms were run on 27×27 cm. sheets of Whatman No. 3 MM filter-paper; 100μl. aliquots of the final concentrates were applied as small circular spots near to one corner of each chromatogram. The chromatograms were developed in batches of eight in frames (Datta, Dent, and Harris, 1950) by upward movement of solvent. Phenol saturated with 0·3 per cent. (w/v) ammonia solution was used as the first developing solvent, whilst the *n*-butanol-acetic acid mixture of Partridge (1948) was used for the second development. The remaining steps in the estimation of the separated amino acids closely followed those described by Fowden (1951), with the exception that the method of spot location on the dried chromatograms (drying at temperatures below 40° replaced ether washing) was altered. This was necessary because the amino acid spots failed to fluoresce satisfactorily at the concentrations employed when viewed under ultra-violet illumination. The difference of behaviour was presumably due to the change from the thin No. 4 paper previously used to the much thicker No. 3 MM paper used in the present investigation.

The spots were located by spraying the chromatograms lightly with a 0·02 per cent. solution of ninhydrin in acetone, followed by heating at 80° until the spots were just visible as faintly coloured areas. These areas were cut out, and the amounts of amino acid present were estimated after quantitative colour development with ninhydrin. When using the thick No. 3 MM paper and very dilute ninhydrin dissolved in the highly volatile solvent acetone, the faintly coloured areas produced on the chromatogram were only clearly visible on the side of the paper actually sprayed. This colour development, performed under conditions which were not optimum for quantitative colour production, would therefore probably only result in the reaction of a very small proportion of the total amino acid present in any spot. In practice this method of location did not appear to lead to any measurable losses.

Triplicate assays were made of the amino acids in each extract. It was found to be advisable to base the assays of asparagine, glutamine, and γ-MG on the determined recoveries of the amides chromatographed under similar conditions, since these recoveries tended to be low. In general the final mean estimates obtained for the amino acids were probably subject to errors not exceeding ±7 per cent.

The amino acids present in the extracts of immature fruits were investigated qualitatively by the above methods of two-dimensional chromatography; the chromatograms were sprayed with a 0·1 per cent. solution of ninhydrin in *n*-butanol and then heated at 100° until maximum colour was produced.

Amino acids in seed and tissue proteins

In contrast with other amino acids only recently demonstrated as occurring in plants (β-alanine and γ-amino-butyric acid), γ-MG and γ-MGA are α-amino acids, and so structural considerations alone would not be expected to prevent their incorporation into protein molecules. It was therefore desirable to obtain both confirmatory evidence for their absence from groundnut seed proteins and new evidence indicating their presence in or absence from proteins present in the vegetative parts of the plants.

Done and Fowden (1952) have shown that γ-MGA alone was stable under the conditions normally used for protein hydrolysis (6 N.HCl for 24 hours at 105°). No evidence was then obtained showing that it was equally stable to these hydrolysis conditions when in the presence of normal plant constituents such as fats and carbohydrates. Lugg (1939) has shown that a number of protein amino acids are partly or wholly destroyed by acid hydrolysis in the presence of smaller amounts of carbohydrate impurities. Since crude preparations of proteins are likely to contain carbohydrate impurity, a demonstration that the hydrolysis products of either whole seeds, petroleum ether extracted seeds, or crude plant proteins did not contain γ-MGA was not considered sufficient proof of its absence from proteins. A demonstration of its absence from enzymatic hydrolysates of the protein fractions was considered to be also essential.

The crude protein fractions were prepared as follows. Repeated petroleum ether extraction of the seeds in an Atomix blender produced on drying a finely divided residue with a total nitrogen content of 9–10 per cent. of its dry weight. This was hydrolysed without further treatment. Crude proteins were prepared from the main vegetative organs (root, hypocotyl, stem, and leaf) by grinding the tissues with a little fine sand and an equal weight of dilute pH 7·2 phosphate buffer until a fairly uniform paste had been obtained. The extract was separated from the sand and tissue debris by expressing through muslin followed by centrifugation. Two volumes of ethanol were then added to one volume of the extract, and the mixture warmed to coagulate the precipitated proteins. These were sedimented by centrifugation, and washed twice by resuspending in small volumes of 70 per cent. ethanol to remove all non-protein amino acids. They were finally washed with ether, dried, and finely ground.

Each protein fraction was then hydrolysed by acidic and enzymatic treatments. 10 mg. of the proteins were hydrolysed as above with 1 ml. of 6N.HCl in sealed ampules. Some humin formation occurred when using seed, leaf, and stem proteins. The hydrolysates were evaporated to dryness *in vacuo* above sodium hydroxide pellets, and the residues dissolved in 0·5 ml. of distilled water. Two-dimensional qualitative chromatograms were run as previously described, using 100 μl. aliquots of each sample. Provided an amino acid constituted more than about 0·5 per cent. of the total protein, its presence would be detected on the chromatograms. Enzymatic hydrolysis was accomplished by utilizing the successive protease and peptidase activities of commercial preparations of papain and erepsin. 10 mg. samples of each protein were treated first with 2 ml. of a saturated solution of papain in dilute pH 7·0 phosphate buffer for 3 days at 37° under toluene. The activity of the papain was destroyed by placing the samples in a boiling-water bath for 15 minutes. 2 ml. of a saturated solution of erepsin at pH 7·0 was then added and hydrolysis allowed to proceed for a further period of 3 days at 37°. After removal of the toluene layer, two volumes of ethanol were finally added to remove the enzymes by precipitation. The supernatants obtained after centrifuging were evaporated to dryness, and the residues treated exactly as those obtained after acid hydrolysis.

RESULTS

Seedling experiments

The nitrogen content of the seeds used was 4·71 per cent. The total nitrogen present in each weighed group of seeds taken for germination could then be calculated and compared with the determined total nitrogen, equal to the sum of the soluble and insoluble nitrogen fractions, present at the times of analysis. Table I shows that the two values are in good agreement at all sampling times. Such agreement indicates that sampling errors are likely to have been small, and that no marked losses of nitrogen occurred during the separation of soluble and insoluble nitrogen fractions. Table I also indicates that the rate of reserve seed protein breakdown was very rapid during the first 7 days of seedling growth, during which the soluble nitrogen increased from 2 to 25 per cent. of the total seedling nitrogen. Thereafter, although the breakdown of protein to soluble nitrogen continued, it did so at a somewhat slower rate. More protein nitrogen was converted to soluble nitrogen in seedlings maintained for 17 days in the dark than in similar seedlings grown in the light.

The rapid increase in the proportion of soluble nitrogen in the initial stages of growth was due mainly to a similarly rapid increase in the α-amino nitrogen, and a marked but smaller increase in amide nitrogen (see Table II and Fig. 2*a*). Both the α-amino nitrogen and amide nitrogen tended to reach maximum values about the 9th day of growth, that is when the initial very rapid rate of protein breakdown had tended to slow down. Although between the 9th and 17th days the levels of α-amino and amide nitrogen tended to remain fairly constant, the levels of ammonia nitrogen present in the seedlings rose continuously at a slightly increasing rate until it became more important than the amide nitrogen.

Fig. 2*b* shows the amounts of these soluble nitrogen fractions expressed as mg. nitrogen per g. total soluble nitrogen. The three fractions constituted a far higher proportion of the total soluble nitrogen present in the seedlings at the 9th day of growth than they did in the ungerminated seeds.

The amounts of α-amino nitrogen present in individual amino acids

TABLE I

Showing the Changes in the Amounts of Soluble and Insoluble Nitrogen Fractions in Seedlings maintained in the Dark for Varying Periods of Time at 25° C.

Growth period (days).	Weight of seeds germinated (g.).	Insoluble nitrogen. (mg.).	Soluble nitrogen. (mg.).	Total determined nitrogen (mg.).	Calculated nitrogen in seeds used (mg.).	$\frac{\text{Total determined N.}}{\text{Calculated N.}} \times 100$	$\frac{\text{Soluble N.}}{\text{Total determined N.}} \times 100$
0	23·73	1,121	23	1,144	1,118	102·3	2·0
3	23·60	984	97	1,081	1,112	97·2	8·9
5	20·85	803	177	980	982	99·8	18·1
7	23·02	798	271	1,069	1,084	98·6	25·3
9	24·92	852	328	1,180	1,174	100·5	27·8
12	22·65	745	311	1,056	1,067	99·0	29·5
14	24·02	745	405	1,150	1,131	101·7	35·2
17	23·23	665	422	1,087	1,094	99·4	38·8
17*	22·94	762	350	1,112	1,081	102·9	31·5

* Grown in light.

extracted from the seedlings grown for different times in the dark are given in Table III, where they are expressed as mg. α-amino nitrogen per g. total nitrogen. Figs. 3*a* and 3*b* express these results in a different way and show the percentage contributions of individual amino acids to the total α-amino nitrogen.

TABLE II

Showing the Amounts of Ammonia, Amide, and α-amino Nitrogen present in the Seedlings maintained in the Dark for Varying Periods of Time at 25°C.

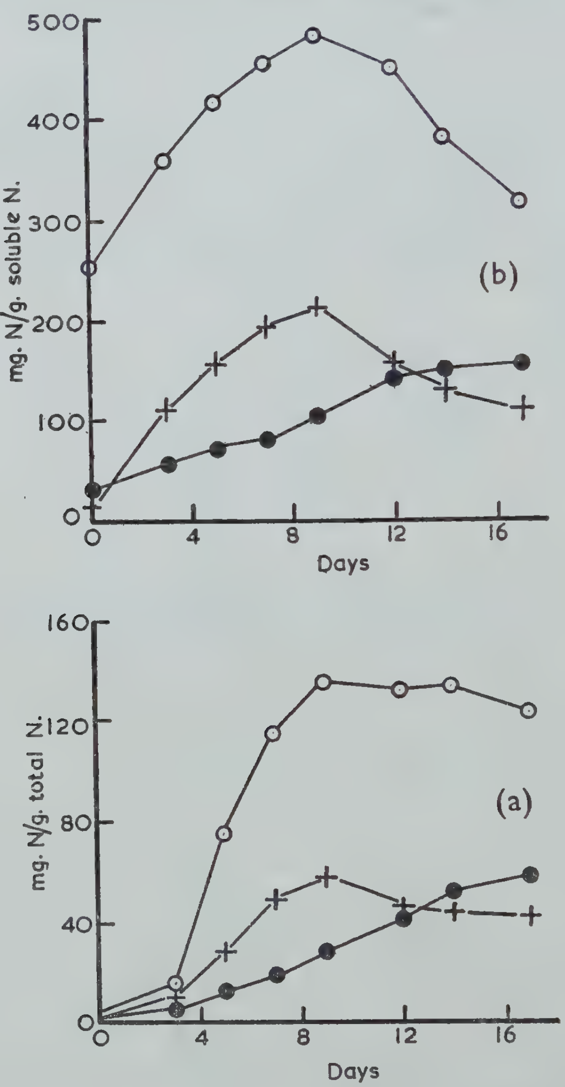
Days.	mg. NH_3-N. per g. total N.	mg. amide-N. per g. total N.	mg. α-amino-N. per g. total N.	mg. NH_3-N. per g. soluble N.	mg. amide-N. per g. soluble N.	mg. α-NH_2-N. per g. soluble N.
0	0·60	0·27	5·1	30	13	254
3	4·8	9·7	31·9	54	109	358
5	12·7	28·1	75·1	70	155	415
7	19·6	48·7	114·4	78	192	452
9	28·6	58·3	135·5	103	210	480
12	41·4	45·6	131·8	140	155	447
14	52·2	45·1	133·0	148	128	378
17	58·6	42·4	122·0	151	109	314
17*	42·7	60·2	116·6	136	191	370

* Grown in light.

No estimates could be obtained for the ungerminated seeds because the chromatograms produced from the soluble extracts were very streaky, due to the presence of large amounts of material other than amino acids. The amounts of all the amino acids and amides tended to increase until about the 7th to 9th day of growth, and so followed the general trend found for the total α-amino nitrogen. After this period glutamine and γ-MG tended to decrease in amount, whilst aspartic acid and asparagine showed further considerable increases (see Table III). Expressed as a proportion of the total α-amino nitrogen (see Figs. 3*a* and 3*b*), certain of these changes became more marked. The dicarboxylic amino acids all tended to increase, although the increase shown by glutamic acid was small. Aspartic acid increased steadily from the 7th to the 17th day, whilst γ-MGA was a fairly constant proportion of the total after an initial sharp increase in the very early stages of growth. The amides behaved somewhat differently. Glutamine progressively decreased during growth, whilst a continuous increase of asparagine occurred. The proportion of the total α-amino nitrogen present was γ-MG passed through a maximum value about the 9th day, and finally dropped to a value of less than half this maximum at the 17th day.

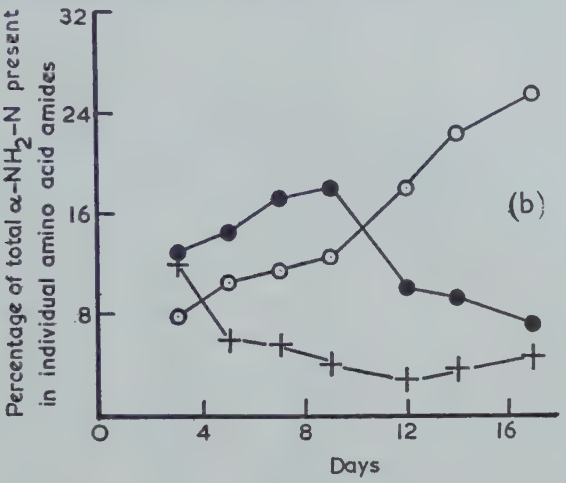
Although the total α-amino nitrogen present in seedlings grown for 17 days in the light was very similar to the amount found for seedlings of the same age grown in the dark, the contributions of individual amino acids and their amides to the total α-amino nitrogen values were markedly different for the two groups of seedlings. The determined levels are shown in the form of histograms in Figs. 4*a* and 4*b*. Large quantities of asparagine and aspartic acid seemed to be characteristic of dark conditions, whilst γ-MGA and γ-MG were far more important constituents in the light.

The distribution of amino acids between the radicles and the remainder of the seedlings (mainly comprised of the cotyledons) was qualitatively investigated at the 7th day of growth. γ-MG was present at slightly higher concentrations in the radicles than in the cotyledons. In contrast only traces of γ-MGA were found in the radicles compared with large amounts present in



FIGS. 2*a* and 2*b*. Showing the amounts of ammonia, amide, and α-amino nitrogen present in seedlings grown in the dark at 25°. ●——● ammonia nitrogen, +——+ amide nitrogen, O——O α-amino nitrogen.

the cotyledons; it was present in the latter organs at a concentration exceeding that of γ-MG. Asparagine, like γ-MG, was also a more important constituent of the radicles than of the cotyledons. Most other amino acids normally present in plants were found in both the radicles and cotyledons, although threonine and tyrosine were present in the cotyledons but could not be detected in the radicles.



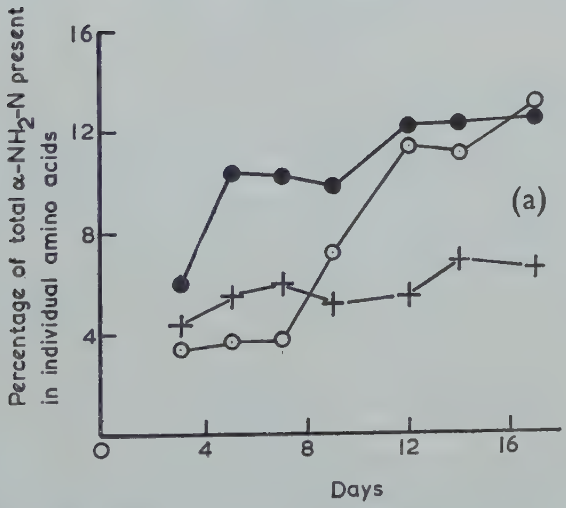


FIG. 3*a*. Showing the percentage contributions of individual acidic amino acids to the total α-amino nitrogen present in seedlings grown in the dark at 25°. ●——● γ-methyleneglutamic acid, ○——○ aspartic acid, +——+ glutamic acid.

FIG. 3*b*. Showing the percentage contributions of individual amides to the total α-amino nitrogen present in seedlings grown in the dark at 25°. ●——● γ-methyleneglutamine, ○——○ asparagine, +——+ glutamine.

γ-MGA could not be detected in an acid hydrolysate of the seed protein fraction, and enzymic hydrolysates also failed to reveal any γ-MG and γ-MGA. No claim is made that the enzymes produced complete breakdown of the proteins to amino acids (compare Damodaran, 1932; Damodaran, Jaaback, and Chibnall, 1932), but all the other normal protein amino acids could be detected in the hydrolysates.

TABLE III

Showing the Amounts of α-amino Nitrogen of Individual Amino acids and Amides present in Seedlings maintained in the Dark for Varying Periods of Time at 25°C.

(Results expressed as mg. α-amino-N. of an amino acid per g. total N.)

Days.	Aspartic acid.	Glutamic acid.	γ-MGA.	Asparagine.	Glutamine.	γ-MG.
3	1·1	1·4	1·9	2·5	3·8	4·1
5	2·8	4·1	7·8	7·8	4·4	10·9
7	4·3	6·9	11·7	13·1	6·4	19·8
9	9·8	7·1	13·4	18·1	5·4	24·5
12	15·1	7·2	16·1	23·8	3·7	13·2
14	14·8	9·2	16·4	29·8	4·9	12·2
17	16·0	8·0	15·3	31·2	5·6	8·7
17*	6·7	5·6	23·5	20·1	2·3	37·8

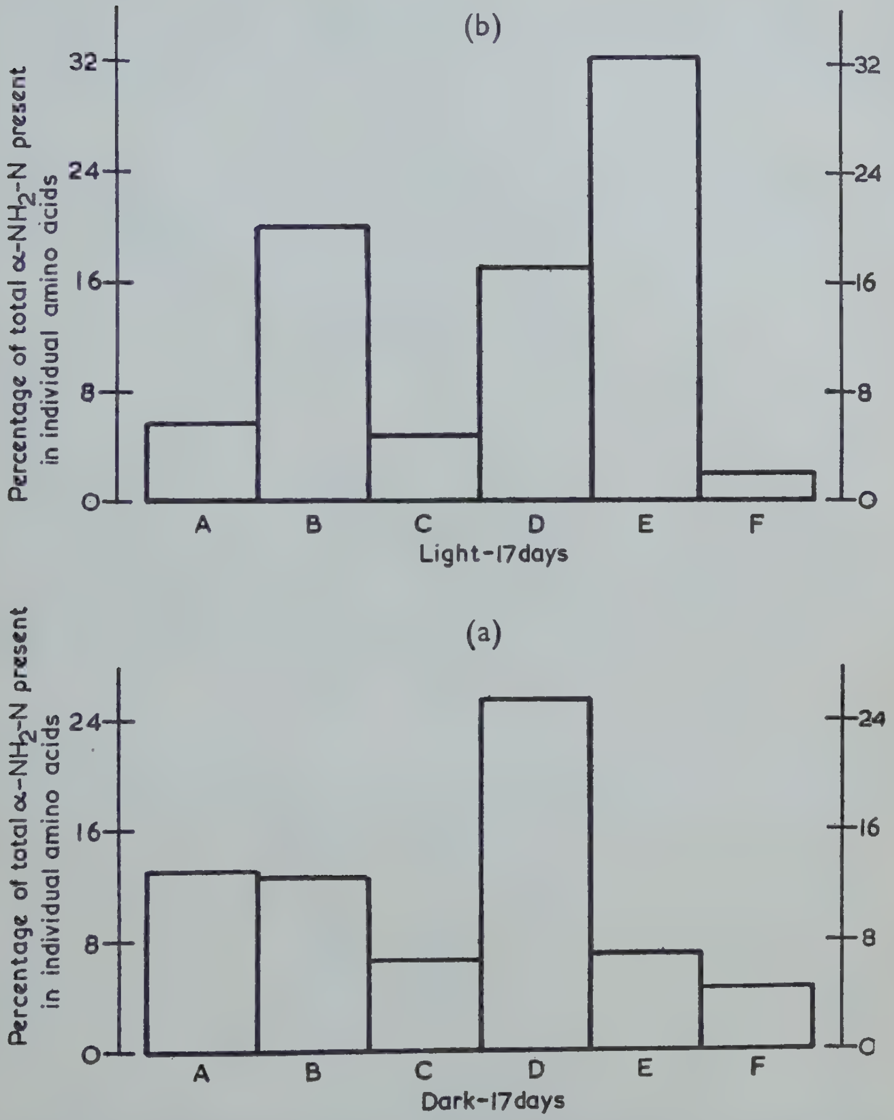
* Grown in light.

Experiments with normal plants

In normal plants (3 weeks old) grown normally in soil the leaves had a much higher nitrogen content on a dry weight basis than the other organs examined, but of this nitrogen only one-fifth consisted of soluble nitrogen compounds, compared with approximately two-fifths or more found as soluble nitrogen in the other tissues (see Table IV). The very high proportion of soluble nitrogen found in the hypocotyls may be related to the fact that they were the most fleshy tissues.

When the distribution of ammonia, amide, and α-amino nitrogen is considered the following points are outstanding. Amide nitrogen was present in large amounts in the roots, stems, and hypocotyls, and in each case represented more than half the α-amino nitrogen. This means that amides were present in amounts greater than the total of all other amino acids. In root and stem tissues over 70 and 80 per cent. respectively of the total α-amino nitrogen was present in amides. Leaves were markedly different from the other organs in that amides constituted only one-quarter of the total α-amino nitrogen. Ammonia nitrogen, however, reached much higher levels than were encountered elsewhere in the plant.

The distribution of the dicarboxylic amino acids and their amides within the plants are shown in Table V, where the amounts present are expressed as mg. α-amino nitrogen in each amino acid per g. total nitrogen. Table VI expresses the determined α-amino nitrogen in each amino acid as a percentage of the total α-amino nitrogen in each tissue.



FIGS. 4*a* and 4*b*. Histograms showing the percentage contributions of individual amino acids and amides to the total α-amino nitrogen present in seedlings grown in the dark and light respectively. (A) aspartic acid, (B) γ-methyleneglutamic acid, (C) glutamic acid, (D) asparagine, (E) γ-methyleneglutamine, (F) glutamine.

TABLE IV

Showing the Amounts of Soluble Ammonia, Amide, and α-amino Nitrogen present in the Main Organs of 3-week-old Plants

Tissue.	Total N content (g. per 100g. dry wt.)	Soluble N (g. per 100g. total N.)	mg. NH_3-N per g. total N.	mg. amide-N per g. total N.	mg. α-NH_2-N per g. total N.	mg. NH_3-N per g. soluble N.	mg. amide-N per g. soluble N.	mg. α-NH_2-N per g. soluble N.
Roots . .	2·30	39·4	21·9	99·6	136·4	55·6	252	346
Hypocotyls .	2·80	58·6	28·7	123·8	214·9	49·0	211	366
Stems . .	3·61	37·9	25·6	112·6	133·8	67·5	297	353
Leaves . .	5·13	19·6	54·4	22·2	90·0	277	113	459

The most notable differences occurred in the distribution of γ-MG and γ-MGA, where the concentration gradients between different tissues were surprisingly large. This can be clearly seen from the histograms shown in Figs. 5*a* and 5*b*. γ-MG, which formed some 60 per cent. of the total α-amino nitrogen in the roots, represented continually decreasing amounts of the total as the farther extremities of the plants were approached. The reverse behaviour was observed for γ-MGA, which could not be detected in the roots but was present to the extent of 40 per cent. of the total amino acids in the leaves. The concentrations of the other amino acids and amides did not vary greatly in the different organs, except that asparagine occurred in much greater amounts in the stem than elsewhere.

TABLE V

Showing the Amounts of α-amino Nitrogen present in Individual Amino Acids and Amides in the Main Organs of Normal 3-week-old Plants

(Results expressed as mg. α-NH_2-N of an amino acid per g. total N.)

Tissue.	Aspartic acid.	Glutamic acid.	γ-MGA.	Asparagine.	Glutamine.	γ-MG.
Roots .	9·2	4·7	0·0	21·1	4·4	81·3
Hypocotyls.	15·7	6·9	4·8	33·9	2·4	92·9
Stems .	7·8	7·8	11·1	51·3	5·6	42·9
Leaves .	3·3	5·3	36·8	12·7	1·5	7·0

TABLE VI

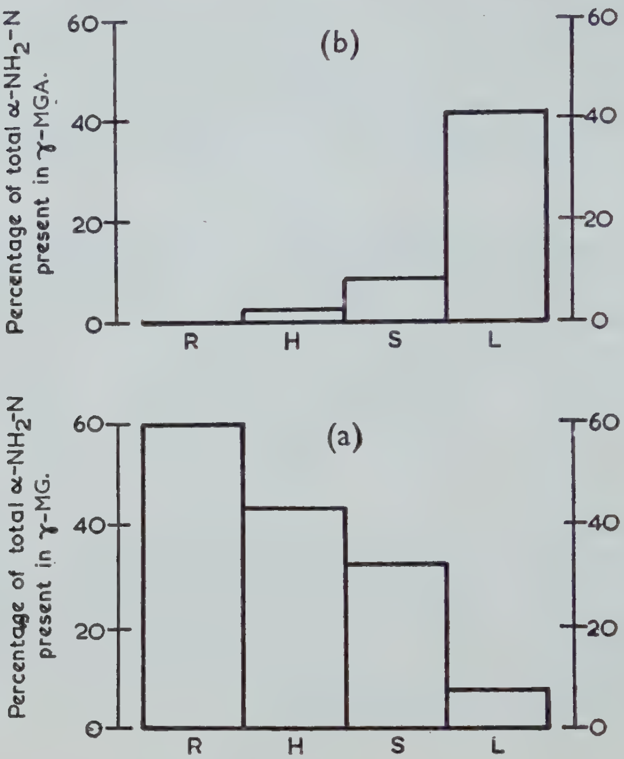
Showing the Amounts of α-amino Nitrogen present in Individual Amino Acid and Amides as a Percentage of the Total Determined α-amino Nitrogen in the Main Organs of 3-week-old Plants

(Results expressed as g. α-NH_2-N of an amino acid per 100 g. total α-NH_2-N.)

Tissue.	Aspartic acid.	Glutamic acid.	γ-MGA.	Asparagine.	Glutamine.	γ-MG.
Roots .	6·7	3·4	0·0	15·5	3·2	59·6
Hypocotyls.	7·3	3·2	2·2	15·8	1·1	43·2
Stems .	5·8	5·8	8·3	38·3	4·2	32·1
Leaves ,	3·7	5·9	40·9	14·1	1·7	7·8

When the amounts of asparagine, glutamine, and γ-MG are expressed as percentages of the total amide nitrogen in the different organs (Table VII), it is seen that the asparagine values tended to increase from one-fifth in the roots to nearly three-fifths of the total in the leaves. γ-MG levels showed the reverse behaviour, whilst the glutamine levels remained at fairly constant but much lower values than the other two amides.

The distribution of nitrogen present in the sap exuding from the stems, after they had been cut across near the points of origin of the lowest leaves, was also determined. Ninety-eight per cent. of the total nitrogen was present



Figs. 5*a* and 5*b*. Showing the distribution of γ-methyleneglutamine and γ-methyleneglutamic acid respectively in the main organs of normal 3-week-old plants. (R) roots, (H) hypocotyls, (S) stems, (L) leaves.

as soluble nitrogen. Ammonia nitrogen only constituted 1·6 per cent. of the total nitrogen, whilst amide nitrogen was 44 per cent., and α-amino nitrogen 46 per cent. of the total. Chromatograms of the sap showed an intense spot of γ-MG, together with barely detectable amounts of other ninhydrin reactive material, and therefore explained the close agreement between amide and α-amino nitrogen figures.

TABLE VII

Showing the Percentages of the Total Amide Nitrogen that are present in Individual Amides in the Main Organs of 3-week-old Plants

(Results expressed as g. individual amide N per 100 g. total amide N.)

Tissue.	Asparagine.	Glutamine.	γ-MG.
Roots . . .	21·2	4·4	81·7
Hypocotyls . .	27·4	1·9	75·0
Stems . .	45·5	5·0	38·1
Leaves . .	57·2	6·7	31·5

No γ-MG or γ-MGA could be detected in either the acidic or enzymic

TABLE VIII

Showing the Amounts of Total, Soluble, Ammonia, Amide, and α-amino Nitrogen present in Roots and Leaves of Different Ages of 8–10-week-old Plants

Serial No. of leaf.	Total N (g. per 100 g. dry wt.)	Soluble N (g. per 100 g. total N.)	mg. NH_3-N per g. total N.	mg. amide-N per g. total N.	mg. α-NH_2-N per g. total N.	mg. NH_3-N per g. soluble N.	mg. amide-N per g. soluble N.	mg. α-NH_2-N per g. soluble N.
1	6·80	15·9	23·6	21·9	54·5	148	138	343
2	4·74	13·9	20·4	10·0	62·4	147	72	449
3	3·20	12·8	23·7	5·6	37·6	185	44	294
4	3·01	11·5	21·3	4·4	38·5	185	38	335
6	2·93	10·0	19·7	8·5	44·6	197	85	446
8	2·68	13·1	37·2	10·7	62·1	284	82	474
10	2·38	11·0	34·8	11·6	48·9	316	105	445
Roots	1·92	32·4	23·3	39·2	128	72	121	394

966.72

hydrolysates of the protein fractions isolated from the roots, hypocotyls, stems, or leaves of these young plants.

A more limited investigation was made of the balance of the nitrogenous constituents in the roots and in leaves of different age of the older plants (8–10 weeks). Ammonia, amide, and total α-amino nitrogen levels in the tissues were again determined, but assays of individual amino acids were confined to γ-MG and γ-MGA. The results are expressed in Tables VIII and IX.

The total nitrogen contents of the leaves were found to decrease with increasing leaf age. The most marked differences occurred during the stage of rapid leaf development (cf. Pearsall, 1931); the differences were considerably greater than those found for many other plant species. The leaves attained their maximum size at about serial number 4. Soluble nitrogen, as a proportion of the total leaf nitrogen, also tended to fall slightly during leaf development. Amide nitrogen levels dropped very rapidly during the stages of leaf development, and then showed a tendency to increase again after maturity was reached. Ammonia nitrogen also increased in older leaves.

TABLE IX

Showing the Amounts of γ-methyleneglutamine and γ-methyleneglutamic Acid as a Percentage of the Total Amino Acids present in Leaves of Different Ages and in Roots of 8–10-week-old Plants

(Results expressed as g. α-NH_2-N in each compound per 100 g. total α-NH_2-N.)

Serial No. of leaf.	γ-MG.	γ-MGA.
1	13·1	14·9
2	5·8	29·7
3	4·7	35·7
4	3·3	26·0
6	4·0	20·5
8	4·6	22·4
10	4·5	18·4
Roots	11·0	0·0

Leaf development was also associated with changes in the relative concentrations of γ-MG and γ-MGA. When these concentrations are expressed as percentages of the total α-amino nitrogen present in the leaves (Table IX), γ-MG was seen to decrease during leaf development, whilst γ-MGA first increased rapidly and then decreased more slowly. This resulted in a change from a system where the amide and acid forms were equally balanced in the very youngest leaves to a state where the acid form was predominant in the other leaves.

The roots of these plants differed from those of young plants in that γ-MG was present in much smaller amounts. Only 11 per cent. of the total α-amino nitrogen was due to γ-MG, compared with 60 per cent. in young plants. Asparagine seemed to be present in approximately equal amounts, whilst γ-MGA was still undetected.

The relative concentrations of the free amino acids and amides in the fruits were determined qualitatively when they had developed to a length of 0·5–1·0 cm., and again when they were 2·0–2·5 cm. long. The immature seeds, hulls, and gynophores were separately harvested and analysed. The results are shown in Table X, the relative concentrations of each amino acid being indicated on an arbitrary scale of 0–5 units; 0 indicates that an amino acid was not detected in the extracts, whilst 5 indicates that it was present in the highest concentrations.

TABLE X

Showing the Distribution of Amino Acids in Extracts of the Fruits at Two Stages of Development

	Fruits 0·5–1·0 cm. long			Fruits 2·0–2·5 cm. long		
	Gynophores.	Seeds.	Hulls.	Gynophores.	Seeds.	Hulls.
Aspartic acid . .	5	5	5	5	4	5
Glutamic acid .	4	5	4	4	4	4
γ-MGA . .	0	0	0	0	0	0
Asparagine . .	3	3	4	3	3	5
Glutamine . .	3	4	2	3	5	3
γ-MG . . .	5	2	4	5	1	2
Serine and/or glycine	3	3	3	2	2	2
Threonine . .	1	1	1	0	2	1
Alanine . .	3	4	3	3	4	4
Valine . . .	0	0	1	1	2	2
Leucines . .	0	0	0	0	1	1
Phenylalanine .	0	0	0	0	1	1
Basic amino acids .	0	1	0	1	1	1

0 represents amino acid not detected in extracts.
1–5 represent increasing concentrations of amino acids in extracts.

Although γ-MG was a predominant constituent of the gynophores, it was only present in small amounts in the developing seeds, whilst γ-MGA was not detected in any portion of the fruits. Aspartic acid, glutamic acid, and their amides, together with alanine, represented the bulk of the free amino acids of each tissue comprising the fruit.

DISCUSSION

When new metabolites are encountered in any plant species, the first essential is to establish, beyond reasonable doubt, their chemical identities. As this has now been done, though the results are unpublished,[1] it is legitimate to consider how or why the plant synthesizes them. Evidence is therefore now presented concerning the possible roles played by the two compounds in the metabolism of groundnut plants. This also indicates, in an indirect manner, pathways by which the compounds may be synthesized.

Synthesis of the new compounds in plants

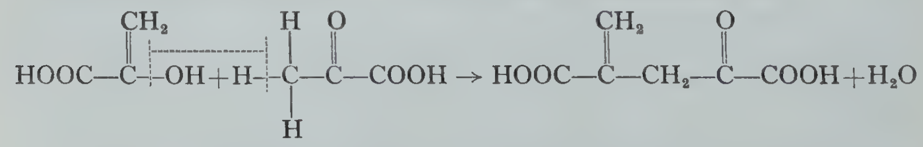
γ-MG and γ-MGA differ from the other dicarboxylic amino acids and their amides in that they are absent from seed proteins. Thus both compounds must have an entirely secondary origin during seedling growth, and their production might be expected to be closely associated with the onset of

[1] See earlier footnote.

respiratory activity within the seedlings. Evidence has been obtained showing that seedlings contain active transaminase enzyme systems capable of forming glutamic acid, alanine, or aspartic acid from mixtures of γ-MGA with α-ketoglutarate, pyruvate, or oxalacetate respectively (Fowden and Done, 1953). Since transamination reactions are normally reversible (Krebs, 1953), there is reason to expect that if the keto acid, γ-methylene-α-ketoglutaric acid, were a normal constituent of the seedlings, then γ-MGA could be formed by a reversal of the previously established reactions. Evidence indicating the probable presence of this keto acid in groundnut seedlings has been obtained (unpublished results, Fowden and Webb),[1] whilst Towers and Steward (1954) have also produced strong evidence in support of its presence in tulips. An alternative pathway of producing γ-MGA from the keto acid may be by a process of reductive amination, similar to that known to exist for glutamic acid formation (von Euler, Adler, Günther, and Das, 1938), but no experimental evidence has so far been obtained in support of such a mechanism.

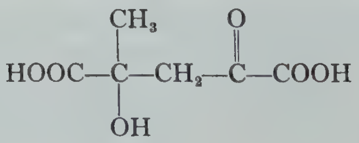
The production of γ-MG presumably follows that of γ-MGA, and may be by a process similar to that resulting in the synthesis of glutamine in plants and involving adenosine triphosphate as an intermediate (see Elliot, 1953; Webster, 1953). Levintow and Meister (1953) have shown that the glutamine synthetase system of peas is not entirely specific to the substrate glutamic acid, and certain glutamic acid derivatives can also be converted to their amides. A similar situation may arise in groundnut seedlings, γ-MG and glutamine being synthesized by mechanisms catalysed by a single enzyme.

More uncertainty surrounds the possible origins of γ-methylene-α-ketoglutaric acid. It is possible that a direct connexion between γ-MG synthesis and respiration exists through the production of this keto acid by respiratory oxidation of carbohydrate or fat. It is very tempting to suggest simply from the standpoint of structural chemistry that the immediate precursor of the keto acid may be pyruvic acid or phospho-enol-pyruvic acid, both of which are formed during oxidative breakdown of carbohydrate. A condensation of two molecules of such acids with the elimination of water or inorganic phosphate could give one molecule of γ-methylene-α-ketoglutaric acid:



$$HOOC-\overset{\overset{\displaystyle CH_2}{\|}}{C}-OH+H-\underset{\underset{\displaystyle H}{|}}{\overset{\overset{\displaystyle H}{|}}{C}}-\overset{\overset{\displaystyle O}{\|}}{C}-COOH \rightarrow HOOC-\overset{\overset{\displaystyle CH_2}{\|}}{C}-CH_2-\overset{\overset{\displaystyle O}{\|}}{C}-COOH+H_2O$$

By purely chemical means salts of pyruvic acid have been shown to undergo a somewhat similar condensation when dissolved in concentration aqueous solution leading to the formation of salts of the following acid (Wolff, 1899):



$$HOOC-\underset{\underset{\displaystyle OH}{|}}{\overset{\overset{\displaystyle CH_3}{|}}{C}}-CH_2-\overset{\overset{\displaystyle O}{\|}}{C}-COOH$$

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[1] Fowden, L., and Webb, J. A. 1954: Biochem. J. lviii, p. iii.

The condensation proceeded at normal temperatures to give good yields of the six-carbon keto acid. Although the product differed from γ-methylene-α-ketoglutaric acid, the fact that condensation occurred under these mild chemical conditions suggests that biological catalysis of a similar type of condensation would probably not be impossible simply on thermodynamic grounds.

Role of the new compounds

Evidence concerning the distribution of γ-MG and γ-MGA can do no more than indicate the *possible* roles of the compounds in the groundnut plant. The available evidence suggests that the two compounds may be important intermediary metabolites formed during the processes which result in a conversion of seed protein to vegetative tissue protein, and that γ-MG is perhaps the main compound in which nitrogen is translocated within the plants.

Although neither γ-MG nor γ-MGA were present in the seed proteins, their concentrations increased very rapidly during the protein breakdown which accompanied seed germination. At the 9th day, when γ-MG levels had reached their maximum, 20 per cent. of the calculated loss of protein nitrogen was accounted for in the nitrogen atoms of this amide and 5 per cent. in γ-MGA. Considerable breakdown of the primary products of protein hydrolysis must therefore occur, possibly via oxidative deamination of the amino acids leading to ammonia, which then enters γ-MGA and γ-MG. Similar partial breakdown of certain amino acids resulting as primary products of proteolysis in seeds of other plants is considered to be a necessary process leading to a redistribution of the total nitrogen amongst the various amino acids before new protein synthesis occurs. The need for such a redistribution is obvious in view of the differences known to occur in the amino-acid compositions of the seed and leaf proteins of some plants. Normally the amides asparagine and glutamine act as compounds in which ammonia nitrogen produced in this way may be temporarily stored. Why the groundnut plant, which contains some 19·2 per cent. of glutamic acid and 5·8 per cent. of aspartic acid in its seed proteins (Guthrie, Hoffpauir, Stansbury, and Reeves, 1949), should choose to synthesize an additional amide to act in this way remains unsolved. The high concentrations of γ-MG in the roots of young plants may largely have been the result of the accumulation occurring during the breakdown of seed protein, since the plants were harvested soon after the seed reserve proteins became exhausted as judged by the withered nature of the residual cotyledons. An equally attractive theory is that the high levels of γ-MG, compared with the other amides, may indicate that the ammonia absorbed and assimilated by these tissues largely entered organic combination through γ-MG initially. The marked decrease in the amounts of γ-MG present in old roots could then be explained as resulting from the decreased rate of ammonia assimilation associated with mature plants. No definite confirmation has, however, been obtained for this mechanism, and further experiments to compare the levels of the amides in young plants grown in the presence and absence of ammonia are desirable.

In the later stages of seedling growth in the dark, asparagine accumulated in much greater amount than γ-MG (see Fig. 3*b*), whose concentration reached a maximum about the 9th day and then showed a progressive fall until the end of the experiment. These later stages of growth would be associated with an increasing degree of depletion of carbohydrate respiratory substrate (Burkhart, 1938, has shown that the large amounts of fat present in groundnut seeds were rapidly converted to carbohydrate during seedling growth), and therefore a tendency for the seedlings to form compounds having a high nitrogen to carbon ratio, i.e. asparagine rather than γ-MG. The composition of the seedlings at the 17th day may then be approaching that associated with their eventual death. No suggestion for a reversal in the concentration of the two amides was observed for seedlings maintained in the light (see Figs. 4*a* and 4*b*). These changes may be compared with those observed by Yemm (1949), who showed that protein breakdown in starved barley leaves led to the production of glutamine when the leaves had a high initial carbohydrate content, and to asparagine when the carbohydrate was initially present at a low level.

A further striking feature of γ-MG distribution was the fact that it was present at high concentrations in the sap exuding from cut stems. Sometimes amounts as high as 3–4 per cent. (w/v) were found to the virtual exclusion of other amino acids and other forms of combined nitrogen. The volume of the sap resulting from a single cut stem was considered to be far greater than that which could have resulted by damage to the cells in the immediate regions of the cut. This view was supported by the observed differences in the amino acids present in the sap and the stem tissue. The sap could be seen to exude principally from the xylem vessels and must be assumed to represent, at least in part, the fluids being translocated up the stem, and γ-MG may be visualized as having an additional role in nitrogen transport in the groundnut plant. Mechanisms whereby both nitrogen atoms of γ-MG could be utilized in the synthesis of new protein are known to exist in the leaves. Unpublished experiments indicate that an active γ-MG deamidase system is present in the leaves leading to the production of γ-MGA and ammonia; little if any similar deamidase activity can be demonstrated in the root tissues. Thus a situation favouring amide synthesis is probably present in the roots (compare the high concentrations of γ-MG with the absence of the acid form in these tissues, Figs. 5*a* and 5*b*), whilst an equilibrium in favour of the acid form would be expected to occur in the leaves (γ-MGA was present in amounts five times greater than γ-MG, Figs. 5*a* and 5*b*). The state of dynamic equilibrium does not appear to have been reached in the youngest leaves to which translocation of the amide would be most active. Ammonia levels were also higher in leaves than in other tissues. The ammonia nitrogen may then enter protein by the accepted channels, whilst the remaining nitrogen atom of γ-MGA could be transferred to other protein amino acids via transamination mechanisms demonstrated to be present in leaves (Fowden and Done, 1953).

Since it is impossible to confirm with certainty that the exuding sap was entirely representative of all the nitrogen containing fluids being translocated in the groundnut plants, there remains the possibility that smaller amounts of other amino acids are also translocated. The composition of the sap from groundnuts is, however, very different from that of the saps exuding from the cut stems of a number of other species examined, not only in that it contains a new amide, but also in that the saps from other species generally contain not one, but several amino acids and amides in moderate amounts, and a number of others in smaller quantities. The similar distribution of amino acids between the radicles and cotyledons of 7-day-old seedlings would indicate that, up to this development stage, translocation involved most of the amino acids resulting from protein hydrolysis in addition to the γ-MG newly synthesized. γ-MG may then become increasingly important in translocation of nitrogen as the plant develops, and begin to be dependent on external sources of nitrogen in place of the reserve seed protein nitrogen.

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The Shoot Apex of some Monocotyledons

II. Growth Organization

BY

MARGARET Y. STANT

(*Botany Dept., University College, London*[1])

With three Figures in the Text

ABSTRACT

In a study of five genera of monocotyledons an attempt has been made to analyse quantitatively the growth organization of the shoot apex. It is suggested that measurement of the proportions

$$\frac{\text{length of rib meristem}}{\text{total length of apex}} \text{ and } \frac{\text{width of flank meristem}}{\text{total width of apex}}$$

gives the best description of the shape of the apex. It is shown that these two ratios are inversely proportional to one another in the plants examined, and the relationship between them is discussed. There is also some correlation between the form and structure of the apex and the growth form of the mature plant.

INTRODUCTION

THE first paper in this series (Stant, 1952) described an investigation into the structure and development of the shoot apices of *Elodea densa*, *Convallaria majalis*, *Carex hordeistichos*, *Luzula sylvatica*, and *Narcissus pseudo-narcissus*. These five genera may be arranged to form a series representing a considerable variation in the size and shape of the apex, ranging from the elongated cylindrical type of *Elodea* to the broad flattened apex of *Narcissus* at the other extremity. An attempt will now be made to analyse further their resemblances and differences. The following analysis is based on measurements of the extent of certain zones within the apex, in order to describe quantitatively the growth organization of the apex.

It has been found that the structure of the shoot apices examined during this work conforms to the tunica-corpus concept of Schmidt. This theory envisages a central corpus of cells dividing in diverse directions and surrounded by an outer envelope of one or several layers of smaller cells, the tunica. The cells of this layer divide anticlinally. Both these meristematic zones are derived from a group of apical initial cells which are larger and divide less frequently than adjacent meristematic cells.

The corpus itself may be further subdivided. There is a central rib meristem zone in which new walls are formed transversely to produce files of partly vacuolated cells. Surrounding the rib meristem, and also derived from the corpus initials, is a cylinder of peripheral or flank meristem which is usually in a highly active state of cell division and takes part in the initiation of new

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[1] Now at Jodrell Laboratory, Royal Botanic Gardens, Kew.

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leaf primordia. It is therefore possible to describe the shoot apex according to a zonation which is based both on the stratification of the growing point and its cyto-histological differentiation. Apices of different genera are found to vary in the amount and proportion of tissue which may be ascribed to these separate zones.

The most salient feature of the apex of *Elodea* (Fig. 1, A) is the long region of rib meristem which occupies a large proportion of the total length of the apex. This is associated with the almost complete absence of flank meristem. Median longitudinal sections show that the rib meristem is directly enclosed in a sheath of uniseriate tunica. In contrast to this, the apex of *Narcissus* (Fig. 1, B) shows a considerable expanse of flank meristem surrounding the rib meristem. The latter appears to occupy only half the total length of the apex, since there is a large mother cell zone at the tip itself, and the corpus is surrounded by three or four tunica layers.

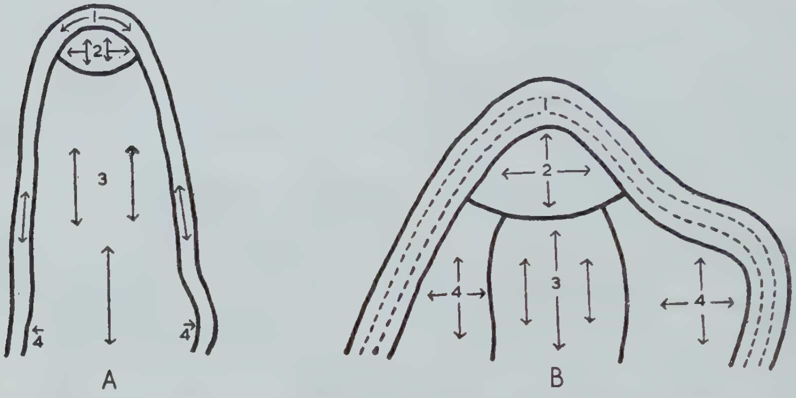

Fig. 1. Diagrams showing zonal structure in the shoot apices of *Elodea* (A) and *Narcissus* (B). Arrows indicate direction of cell divisions. 1, tunica and its initials; 2, corpus initials; 3, rib meristem; 4, flank meristem.

Since these two zones, the rib and flank meristems, are the agents responsible for increase in length and diameter of the apex respectively, the establishment of a relationship between them would be some contribution towards a mathematical interpretation of apical growth and form. In an attempt to do this it was decided to measure the extent of the rib and flank meristem zones in all the apices, and to try and correlate the results with the shape of the apex as a whole.

A Quantitative Analysis of Apical Zonation

With the aid of a micrometer eyepiece, the following measurements were taken from median longitudinal sections of each species:

L. Total length of apex from the tip to the base of the first leaf primordium.
B. Total width of apex, including first leaf primordium.

R. Length of rib meristem.

F. Total width of flank meristem (sum of lateral corpus and tunica on each side).

It was not always easy to determine exactly the total length of an apex since there is always a gradation between the meristematic tissue of the growing point and the vacuolated parenchyma of the stem axis. For this reason *L* was defined as finishing at the base of the first primordium. This also made the results for different species more comparable. The same difficulties were encountered in measuring *R* and *F*, so that a considerable personal error in measuring is inevitable.

TABLE I

Dimensions of Growing Points

	Elodea.	*Convallaria.*	*Luzula.*	*Carex.*	*Narcissus.*
L . .	214·1 (17·48)	215·1 (29·21)	133·4 (24·84)	77·3 (15·18)	240·8 (57·96)
B . .	143·8 (14·95)	209·1 (22·31)	166·9 (26·22)	109·4 (33·12)	399·9 (68·31)
R . .	154·0 (8·51)	142·1 (15·87)	74·66 (16·1)	39·08 (12·88)	101·3 (23·23)
F . .	44·61 (6·9)	86·57 (12·657)	76·53 (8·97)	57·66 (6·9)	224·7 (35·19)
L/B . .	1·5634 (0·1801)	1·0296 (0·0949)	0·8220 (0·1185)	0·7226 (0·1108)	0·6039 (0·1384)
R/L . .	0·7211 (0·0316)	0·6635 (0·0425)	0·5605 (0·0772)	0·4954 (0·0684)	0·4214 (0·0518)
F/B . .	0·3257 (0·0571)	0·4101 (0·0425)	0·4735 (0·0783)	0·5392 (0·1016)	0·5767 (0·1391)

A summary of the results is given in Table 1. Each figure is the arithmetic mean of approximately 10 readings from different apices of that species. The standard deviation(s) was calculated for each result and is the figure given in brackets immediately below. The units are milli-microns (μ = 10—6 metre).

Reference to Table 1 will show that the simple measurements for each genus are not significant. The apex of *Narcissus* is longer than that of *Elodea*: it is altogether a larger apex and its unit length is not a true indication of its relative elongation. However, when these results are calculated as the ratios *L/B*, *R/L*, and *F/B*, they fall into an orderly series. *L/B* gives a good measure of the shape of the apex and it is clearly seen that *Elodea* has the highest *L/B* and *Narcissus* the lowest. The other species range between these two extremes. Similarly, the different apices can be arranged in the same descending order for the proportion of rib meristem (*R/L*), but the proportion of flank meristem increases inversely as *R/L* decreases.

These relationships and their correlations are shown more clearly in Fig. 2. They indicate that the proportion of rib meristem and the width of flank meristem are the main factors in describing the shape of the apex, as measured by the length/breadth ratio. *Narcissus* with the lower L/B ratio has a high proportion of flank meristem and relatively little rib meristem. At the opposite end of the series is the long, narrow growing-point of *Elodea* with a high proportion of rib meristem and very little flank meristem. The other species are situated at intermediate points on the curves R/L and F/B.

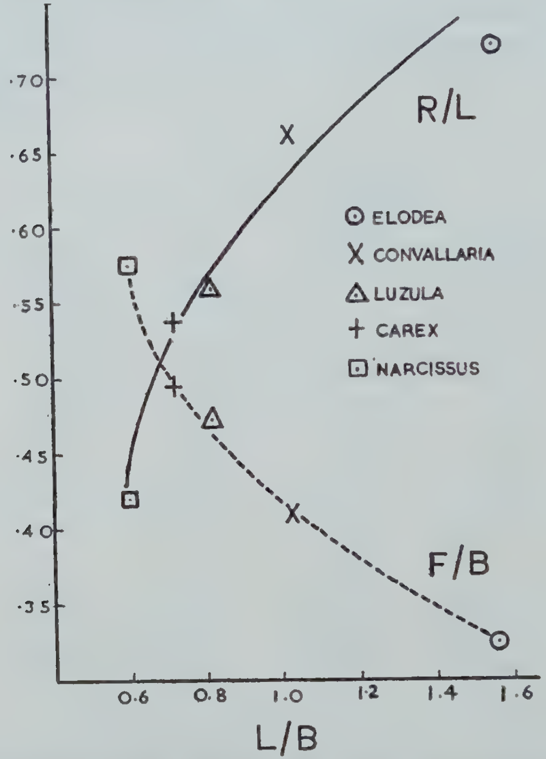

FIG. 2. The relationship between two proportions within the apex and its shape. L, total length of apex; B, total width of apex; R, length of rib meristem; F, width of flank meristem.

Fig. 3 shows that when the proportion of rib meristem is plotted against the proportion of flank meristem (R/L against F/B), a straight line is obtained. $R/L \propto \frac{1}{F/B}$ for the apices of all the different species examined. The relative amounts of rib meristem and flank meristem are thus intimately correlated with the shape of the shoot apex, and these two ratios are inversely proportional to one another.

It seems possible that some nutritional factor may be involved in this relationship. The extent to which any meristem or particular zone of meristem can develop must be determined to some extent by the amount of food substances reaching the dividing cells. It has been noted in the first paper in this series that the cells of the flank meristem appear to be in a very active state of division, and they would therefore act as a considerable drain on the food material passing into the apex. It would seem reasonable to suppose that the amount which they consume would be proportional to the extent of the tissue or the width of the apex which they occupy, and thus inversely proportional to the amount which is left to diffuse onwards through the rib meristem. This lateral diversion of foodstuffs into the flank meristem may control the extent to which the rib meristem is able to develop, and can be used as a working hypothesis to account for the mathematical correlation between the two ratios R/L and F/B.

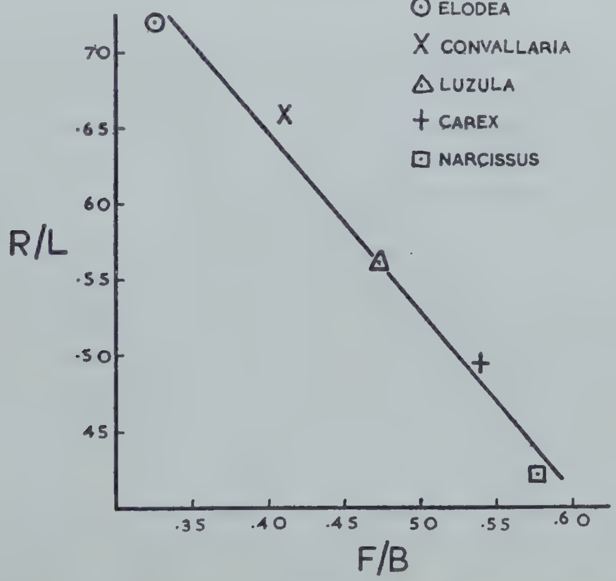

FIG. 3. The relationship between two proportions within the apex. L, total length of apex; B, total width of apex; R, length of rib meristem; F, width of flank meristem.

An alternative formulation of the same idea suggests that the extent of the rib meristem may depend on its diameter in relation to the total width of the apex. For the relative diameter of the rib meristem would be a measure of the food material passing up through the base of this region and must bear an inverse relationship to the relative diameter of flank meristem.

This type of analysis of ratios within the apex is perhaps a rather superficial attempt to solve the problems connected with the form of the apex, but it is clearly only a beginning. It explains how differences in the shape of the shoot apex may be related to differential growth of the zones within the apex, and it is suggested that the relationship between these may have a nutritional basis. This does not elucidate the basic problem of how these differences arise in the first place; it is merely a statement of the mechanical factors involved. As Wardlaw (1952) has suggested in his recent extensive survey, the final resolution of such morphogenetic problems will require the collaboration of workers in diverse biological fields.

CORRELATION WITH THE GROWTH FORM OF THE MATURE PLANT

Depth of flank meristem.—Differences in the proportion of flank meristem *F/B* present indicate that differences in massiveness of the mature leaves of a plant may be initiated at the apex. *Elodea* has extremely small delicate leaves consisting of only two layers of cells, the upper and lower epidermis, except in the region of the midrib, where there is some development of mesophyll.

Leaf initiation in *Elodea* has been described in an earlier paper. The primordium is derived almost entirely from a number of tunica cells which divide anticlinally to form the upper and lower epidermis. The small amount of mesophyll develops from three cells cut off from the outer layers of the corpus behind the primordium. Since the corpus consists predominantly of rib meristem, *Elodea* has a very low *F/B* ratio.

This contrasts with the condition in *Narcissus*, which by observation has more massive leaves than the other four species. Here a considerable part of the corpus tissue is concerned with leaf formation and only a small central portion consists of rib meristem. A high proportion of flank meristem is, in this instance, associated with leaves of some considerable size.

Length/Breadth ratio of the apex. Again it is simpler to consider the two extreme cases first. The mature plant of *Elodea* consists of an elongated stem axis bearing whorls of small leaves at intervals along its length. There is a considerable development of the internodes in this aquatic plant. The elongated mature form is paralleled in the shoot apex from which it develops. The apex has a higher *L/B* ratio than any of the others studied. This is coupled with a high proportion of rib meristem, the tissue which is responsible for growth in length and the initiation of internodes. The leaf primordia are formed on the sides of the long cylindrical growing-point and are pushed farther back and apart by the activity of the files of vacuolating dividing cells. The later elongation of the internodes is brought about by increased vacuolation and cell extension. This may be compared with the grass apices examined by Sharman (1947), where the cylindrical type of apex is again associated with an elongated mature form and the development of internodes.

Narcissus has a very abbreviated stem axis which is more typical of the monocotyledons as a group. The young leaf primordia of the shoot apex are closely ensheathed within the older leaves which arise farther out on the flattened stem axis, and are thus inserted at nearly the same level. This correlates with a shoot apex of similar form in which a wide, flat, broadly conical growing-point is surrounded by a few leaf primordia which arise at about the same level on the growing-point, the older ones being farthest from the centre. This absence of the development of internodes is associated with a very low proportion of rib meristem.

Between these two extremes lies *Convallaria*, whose L/B and R/L ratios are much less than *Elodea* but higher than the other three species. As might be expected, the mature plant shows some development of internodes. There is a short stem axis on which the circular leaves are inserted one above the other.

It therefore seems that trends in the shape and structure of the growing point are repeated in the form of the adult plant, which develops as a result of apical activity and subsequent differentiation. This is a short statement of simple and obvious facts, but it has not been referred to in previous literature on the subject and it is briefly mentioned here as a possible line for future consideration and investigation.

SUMMARY

1. Brief reference is made to the characteristic features of the zonation of the shoot apex in five genera of monocotyledons.

2. From median longitudinal sections measurements were made of the length and breadth of the apices and the extent of the rib and flank meristems in the different genera.

3. The results tabulated are given in the form of the arithmetic mean together with the standard deviation.

4. The ratios $\frac{\text{length of rib meristem}}{\text{total length of apex}}$ and $\frac{\text{width of flank meristem}}{\text{total width of apex}}$ have been calculated and are plotted against the length/breadth ratio of the whole apex for each species in Fig. 2, and against one another in Fig. 3.

5. The correlations shown indicate that the proportions of rib meristem and flank meristem are the main factors in describing the shape of the apex.

6. The two ratios R/L and F/B are inversely proportional to one another. This relationship may have a nutritional basis.

7. It is suggested that the proportion of flank meristem may influence the size and form of the final leaf.

8. The shape of the apex shows some correlation with the life form of the mature plant.

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Experimental and Analytical Studies of Pteridophytes

XXIV. Investigations on *Marsilea*

4. ANATOMICAL EFFECTS OF CHANGES IN SUGAR CONCENTRATION

With Plates XXIII–XXV and three Figures in the Text

BY

A. ALLSOPP

(*Department of Cryptogamic Botany, University of Manchester*)

ABSTRACT

Sporelings of *Marsilea*, grown aseptically in various concentrations of sugars, particularly glucose, have been investigated anatomically.

It is shown that, corresponding to the features of the external morphology, the anatomy of the sporelings from the lower sugar concentrations (1 and 2 per cent. glucose) has many characteristics of the water forms of amphibious plants, while that of the sporelings from the higher sugar concentrations (4 and 5 per cent. glucose) has the characteristics of typical land forms.

A number of the differences between the two types of sporeling may be referred to a change in the duration and direction of cell-division in the rhizome-apex. Views as to the factors influencing the origin of land or water forms are briefly discussed.

INTRODUCTION

IT has been shown in a previous contribution (Allsopp, 1953*a*) that the heteroblastic development and external morphology of sporelings of *Marsilea* growing in aseptic culture are profoundly affected by changes in the sugar concentration of the medium. At low sugar concentrations, e.g. 1 and 2 per cent. glucose, the sporelings have the characteristic feature of a typical water form, while at high sugar concentrations, e.g. 4 and 5 per cent. glucose, the sporelings correspond in external morphology to the normal land form, even though they remain completely submerged.

In view of the bearing of this work on hypotheses as to the causal factors responsible for the marked dimorphism of amphibious plants, it was considered desirable to extend the morphological observations by a study of the anatomical responses to changes in sugar concentration. The present paper deals with the results of this anatomical study.

MATERIALS AND METHODS

The sporelings used were those whose external morphology has already been described in the previous paper of this series (Allsopp, 1953*a*). Representative sporelings were fixed in formalin-acetic-alcohol immediately after

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removal from the culture tubes. Sections were cut 10μ thick and stained in safranin followed by Delafield's haematoxylin.

Owing to the delicacy of the tissues in the cultured plants, direct observations could be made of the epidermal cells of the leaves and roots after decolorization in alcohol. Epidermal cells of stem and petiole were drawn from serial sections. All drawings of cells were made using the camera lucida.

The previous study of the effect of sugar concentration on external morphology had shown that sporelings grown in culture solutions containing either glucose, fructose, or sucrose were virtually identical if approximately isosmotic solutions were compared. After examination of the leaves had shown that this correspondence in external morphology was paralleled by a close similarity in anatomical details, further investigation was restricted to the forms developing in the different glucose concentrations.

The initial comparison was made between 'water' and 'land' forms, from 1 and 5 per cent. glucose respectively, and revealed such striking anatomical differences that a further comparison was made between forms, from 2 and 4 per cent. glucose, obtained from a batch of cultures (MD8/5) which were started 7 months after the first set of cultures. The results obtained with the second set of cultures provide ample confirmation of those from the first batch.

ANATOMICAL OBSERVATIONS

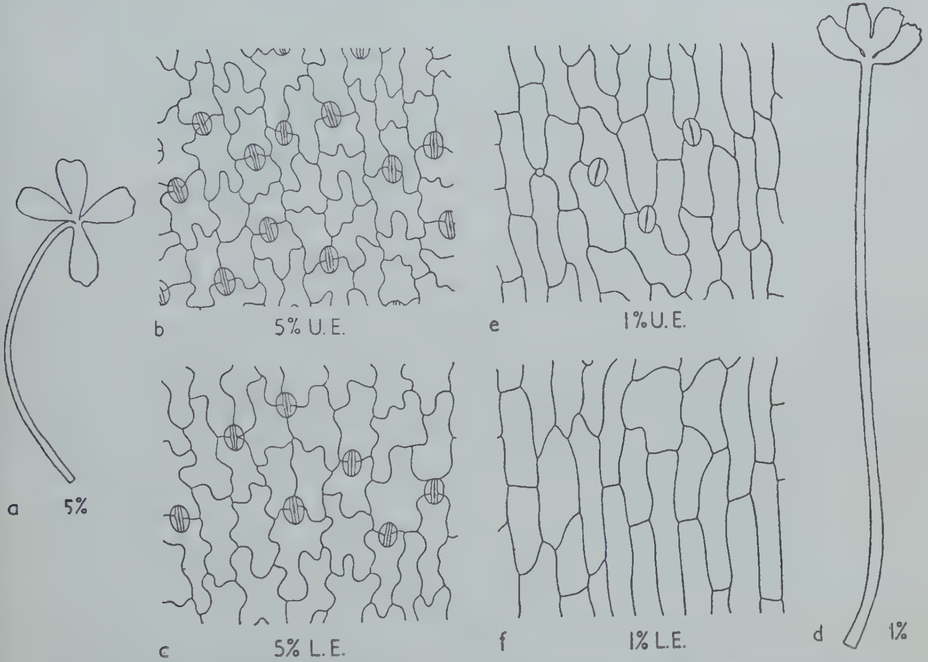
1. *The leaf*

In the account of the effect of sugar concentration on external morphology it was shown that leaves from plants grown in the lower sugar concentrations (1 and 2 per cent. glucose, 1 per cent. fructose, 1 per cent. sucrose) differed markedly from those from the higher sugar concentrations (4 and 5 per cent. glucose, 5 per cent. fructose, 5 per cent. sucrose). In the lower concentrations the leaves had each a stout petiole with a relatively small lamina lying in the plane of the petiole, whereas in the higher concentrations the leaves had a thin petiole with the divisions of the lamina expanded in a plane almost perpendicular to the petiole. In the anatomical study of the two types of leaf investigations have been made of both lamina and petiole.

(*a*) *Lamina.* Although the anatomy of the leaf of *Marsilea* has not been described in detail (Tournay, 1951), it has been known since the work of Hildebrand (1870) that the normal aerial leaves bear numerous stomata on both surfaces, whereas the floating leaves, which arise either following submergence of mature plants or during normal development of the sporeling, have stomata only on the upper surface. Hildebrand also showed that while in the aerial leaves the stomata are sunken below the general level of the surface, and partly covered by overgrowth of the surrounding cells, in the floating leaves the stomata are not sunken and are even rather higher than the level of the epidermis. These observations were confirmed by Senn (1909). It is also known (Sadebeck, 1902) that the juvenile leaves appearing during the normal development of the sporeling bear stomata even when completely submerged. The stomata of the juvenile leaves, as of the floating leaves, are restricted to the upper epidermis.

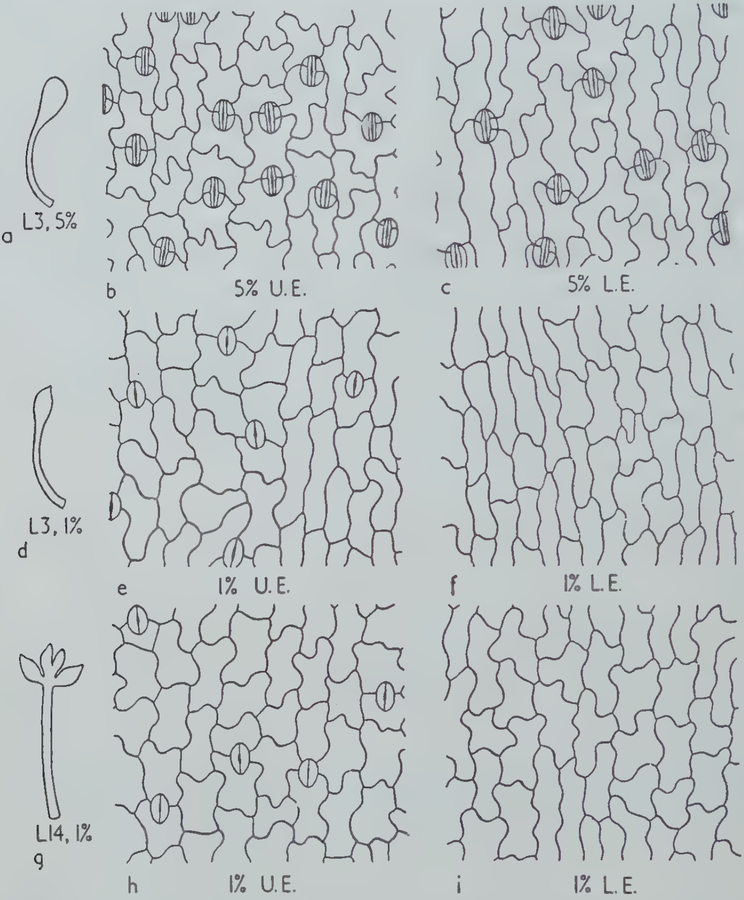
There is thus a marked variability in stomatal distribution in response to environmental conditions. In the present work equally distinct differences in stomatal distribution have been found in sporelings from low and high sugar concentrations.

Text-figs. 1*a*, *b*, and *c* illustrate a typical quadrifoliate leaf from a sporeling grown in a 5 per cent. glucose medium, together with outline drawings of cells from the upper and lower epidermis of the central region of one of the proximal leaflets, while Text-figs. *d*, *e*, and *f* illustrate the corresponding features of a quadrifid leaf of similar age from a sporeling grown in 1 per cent. glucose. It is at once evident that at the higher sugar concentration both upper and lower epidermis bear numerous stomata while at the lower sugar concentration the stomata are fewer on the upper epidermis and entirely lacking on the lower epidermis. This result has been confirmed in a considerable number of independent experiments. The differences in stomatal frequency in the two types of leaf are due almost entirely to changes in stomatal index, as defined by Salisbury (1927), and not merely to changes in cell size.



TEXT-FIG. 1. *M. Drummondii*. Leaves of similar age from sporelings grown in media containing (*a*) 5% glucose, (*d*) 1% glucose. (×7.) (*b*) and (*c*), upper and lower epidermis of (*a*); (*e*) and (*f*), upper and lower epidermis of (*d*). (×160.)

Apart from the stomatal distribution other differences are also observed in the epidermis of leaves of the diverse forms. Thus each guard cell of the aerial or of a 'high sugar' leaf has a prominent line running parallel to the stomatal aperture (Text-figs. 1*b* and *c*). This appearance is produced by an overgrowth of the surrounding epidermal cells and is accentuated by a marked cuticular thickening of the free region of the guard cell. In a leaf from the lower sugar concentrations there is neither overgrowth by the epidermal cells nor any marked thickening of the cuticle of the guard cells. Consequently the guard cells lack the prominent lines found in the higher sugar concentrations (Text-fig. 1*e*).



TEXT-FIG. 2. *M. Drummondii.* (*a*) and (*d*), third leaves of sporelings grown in media containing 5 and 1% glucose respectively; (*g*), first quadrifid leaf (L 14) of sporeling from 1% glucose. (All ×7.) (*b*) and (*c*), (*e*) and (*f*), (*h*) and (*i*), upper and lower epidermis respectively of (*a*), (*d*), and (*g*). (×160)

The features described above for 'land' forms from 5 per cent. glucose and 'water' forms from 1 per cent. glucose have been found also in 'land' leaves from 4 per cent. glucose, 5 and 7·5 per cent. fructose, 5, 7·5, and 10 per cent. sucrose, and in 'water' leaves from 2 and 3 per cent. glucose and 1 per cent. fructose and sucrose. Similar effects have been obtained with *M. vestita.*

The account given above refers to leaves of the mature quadrifid or quadrifoliate type. Examination has also been made of the leaves appearing at successive stages of sporeling development. Thus the early leaves from sporelings grown in 1, 2, 3, and 5 per cent. glucose, as illustrated in Allsopp (1953*a*), Text-fig. 1, were investigated. In all cases the non-laminate acicular cotyledon bore only occasional irregularly scattered stomata, while the spatulate, bifid, and quadrifid juvenile leaves from the lower (1, 2, and 3 per cent.) glucose concentrations had stomata on the upper epidermis only (Text-figs. 2*d*, *e*, *f*, *g*, *h*, and *i*). This distribution of the stomata corresponds to that previously recorded for juvenile leaves of *Marsilea*. But the condition was very different in the juvenile leaves from the 5 per cent. glucose. In this case both surfaces of the lamina of even the earliest spatulate leaves bore numerous stomata (Text-figs. 2*a*, *b*, and *c*), which, as in the adult leaves, were sunken, with marked thickening of the free surface of the guard cells.

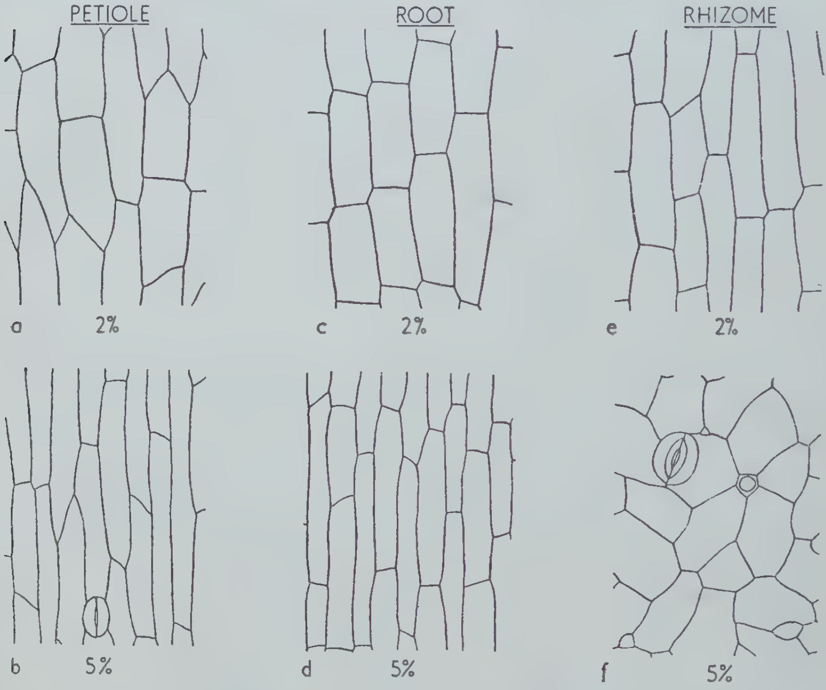
Reference should also be made to the wavier outline of the epidermal cells from the higher sugar concentrations, which is found in both juvenile and adult leaves (Text-figs. 1*b* and *c*, Text-figs. 2*b* and *c*), but this distinction is neither so marked nor so constant as the features already discussed.

The pronounced differences in epidermal characteristics find a parallel in the mesophyll and vascular tissue of the leaf. Previous accounts of these tissues are very inadequate. The structure of the submerged water leaves of *Marsilea*, which resemble those of sporelings from low sugar concentrations (Allsopp, 1953), has never been described, while accounts of the aerial leaves (Russow, 1872; Senn, 1909; Ogura, 1938) are also incomplete but refer to the presence of a palisade layer with a lacunar parenchyma below.

A transverse section of the leaflet of a sporeling from 5 per cent. glucose solution (Pl. XXIII, Fig. 1) reveals a structure similar to that of an ordinary foliage leaf. The upper and lower epidermis are entirely or almost without chlorophyll, contrasting markedly in this respect with the layer of palisade cells below the upper epidermis. The section also shows that the stomata are appreciably sunken below the level of the over-arching epidermal cells, and that the guard cells have strongly thickened walls with internal and external cuticular ridges. The vascular bundles are well developed with thickened lignified xylem and a prominent endodermis. Serial sections of a leaflet reveal that the pulvinus at the base is composed of uniform approximately isodiametric cells fitting closely together with only small intercellular spaces at the angles. This structure is similar to that encountered in the pulvini of a considerable range of plants (Haberlandt, 1914).

A section of the lamina of a leaf from 1 per cent. glucose is very different in appearance (Pl. XXIII, Fig. 2). There is no palisade layer, the mesophyll consisting entirely of loosely fitting cells separated by wide intercellular spaces and connected by tubular prolongations. Furthermore, the mesophyll comprises only 4 layers of cells as opposed to the 6 layers found in the leaf from 5 per cent. glucose. Both upper and lower epidermis are as well supplied with chloroplasts as the cells of the mesophyll. The stomata, restricted to the upper epidermis, are not sunken and show no pronounced thickening of the guard cells, while the vascular bundles are smaller in cross-sectional area, with the xylem less thickened and little lignified. There is no pulvinus and the air-spaces of the petiole continue without interruption into those of the leaf.

Sections taken from the sporelings from 4 and 2 per cent. glucose (Pl. XXIII, Figs. 3 and 4 respectively) show similar differences, although the spongy mesophyll is rather less lacunar in the leaf from 4 per cent. glucose than in that from 5 per cent.



Text-fig. 3. *M. Drummondii.* Epidermal cells of (*a*) petiole, (*c*) root, and (*e*) rhizome internode of sporeling from 2% glucose medium. (*b*), (*d*), and (*f*), corresponding cells of sporeling from 5% glucose medium. (×205.)

(*b*) *Petiole.* It has already been pointed out that the petioles from the high sugar concentrations are always thinner and frequently longer than those from low sugar concentrations. This distinction is accounted for by the anatomical details of the two types of petiole.

A comparison of a transverse section of a petiole from 5 per cent. glucose (Pl. XIII, Fig. 5) with one from 1 per cent. glucose (Pl. XIII, Fig. 6) shows that the large cortical air-spaces are much more prominent in the latter. The partitions separating these air-spaces show only 2–3 radially arranged cells in the section from 5 per cent. glucose, while in the section from 1 per cent. glucose the partitions show 10–12 cells. The vascular tissue is considerably better developed in the petiole from 5 per cent. glucose. There are more xylem tracheides, which are also wider, thicker-walled, and more strongly lignified. The outer cortical and the epidermal cells, on the contrary, are appreciably larger in 1 per cent. glucose.

In the subsequent experiment with 4 and 2 per cent. glucose solutions the petioles showed exactly the same differences (Pl. XXIII, Figs. 7 and 8).

In longitudinal sections of the petioles the cortical and epidermal cells from the two higher sugar concentrations (4 and 5 per cent.) appear more elongated, as the ratio of length to breadth is substantially greater, but the absolute length of the cells is not increased in proportion, and is only slightly higher than in the two lower sugar concentrations. This relationship between the dimensions of the epidermal cells is illustrated by cells from the middle regions of the petioles from 2 and 5 per cent. glucose in Text-figs. 3*a* and *b*.

2. *The root*

External observation had already shown that in the higher sugar concentrations the roots are much longer, more slender, and more branched than in the lower sugar concentrations. These differences in external appearance are accompanied by considerable differences in anatomy.

The roots from the two lower sugar concentrations (1 and 2 per cent., Pl. XXIII, Figs. 10 and 12) show a remarkably regular construction. Below the piliferous layer is a single layer of radially elongated closely fitting cells. Within is a highly lacunar tissue whose cells are connected radially by delicate tubular prolongations but are separated laterally by large air-spaces so as to form longitudinally running partitions. These partitions are attached opposite every alternate radial wall of the outer cortical layer; hence the cells of the latter are twice as numerous as the partitions. The partitions are also connected laterally in pairs so that smaller triangular air spaces alternate with larger oval ones. Smaller intercellular spaces are found in the inner cortex where the cells are connected both radially and laterally. This characteristic structure of the cortex corresponds closely with the figure and description given by Russow (1872) for *Pilularia globulifera*, whose roots were said to be very similar in anatomy to those of the water form of *Marsilea aegyptiaca*.

The cortex of the roots from the higher sugar concentrations (5 and 4 per cent., Pl. XXIII, Figs. 9 and 11) presents a very different appearance. Large air-spaces are lacking and all the cells are in lateral contact. There are fewer layers of cells and the hypodermal layer is not markedly differentiated. The larger roots, however, show a distinct thickening of the walls of the two inner cortical layers, immediately outside the endodermis. The cortex of the roots from the higher sugar concentrations is thus similar to that described by Russow for the roots of the normal land form of *M. Drummondii*.

The steles of the roots from the various sugar concentrations show little variation in size, and consequently form a greater proportion of the cross-section in the high than in the low sugar concentrations. The xylem of the roots from the high sugar concentrations is thicker-walled and more strongly lignified.

Examination of longitudinal sections shows that although the roots from the higher sugar concentrations are very much longer and narrower than those from the low concentrations, their cells are only slightly longer, but are appreciably narrower. This relationship is illustrated for the cells of the piliferous layer of roots from 2 and 5 per cent. glucose in Text-figs. 3*c* and *d*.

3. *The rhizome*

Sections of the rhizome reveal that as in the root and leaf there are considerable structural differences between the materials from the various sugar concentrations.

Pl. XXIV, Figs. 13, 14, 15, illustrate transverse sections of rhizomes from 1, 2, and 4 per cent. glucose respectively. The rhizome from 5 per cent. glucose, not illustrated, is indistinguishable from that from the 4 per cent. solution.

The general constructional plan is the same in the different sugars, but the air-spaces of the middle cortex are far larger in the two lower sugar concentrations. Sections from 4 and 5 per cent. glucose show only 2 or 3 radially arranged cells in the longitudinal partitions separating the air-spaces, while in 1 and 2 per cent. glucose the partitions show there are 8–10 cells. The number of layers of cells in the inner and outer cortex, however, is not appreciably different in the various solutions.

As illustrated by Pl. XXIV, Fig. 15, the stele is larger in rhizomes from the higher sugar concentrations and stands out more prominently from the surrounding tissues. In the lower sugar concentrations the tracheides are thinner-walled and less strongly lignified, and for the most part are arranged in a single tangential series, in contrast with the condition in the higher sugar concentrations, where there are frequently three contiguous layers of tracheides.

The differences between the rhizomes from the low and high sugar concentrations as for the leaves and roots are apparently similar to those between land and water forms of *Marsilea*. No detailed comparison has been made previously, but Russow (1872) refers to the occurrence of large lacunae in the cortex of the water form of *M. aegyptiaca*. His figure of the vascular cylinder of this water form is very similar to the vascular tissues of the rhizomes from low sugar concentrations. It is of interest to note that Johnson and Chrysler (1938) found similar differences between submerged and emersed regions of the rhizome of *Regnellidium*.

The epidermal and cortical cells of rhizomes from the 1 and 2 per cent. sugar solutions are elongated and arranged in regular longitudinal series, in contrast with the corresponding cells from 4 and 5 per cent. glucose solutions, which are wider, shorter, and more irregularly arranged (see Text-figs. 3*e* and *f*). The dimensions of the cells do not account for the difference in internode extension in the two series, for the cells from the lower sugar concentrations are only two or three times the length of those from the higher concentrations, while the internodes show much greater differences in length. In other words, the internodes of the sporelings from low sugar concentrations have many more cells in the longitudinal direction than are found in the internodes from the higher sugar concentrations. In contrast, as shown by the transverse sections, the number of cells in the radial and tangential directions is substantially the same in the two sets of sugar concentrations.

4. *The rhizome apex*

Longitudinal sections of the apices from 1 and 5 per cent. glucose are illustrated in Pl. XXIV, Figs. 16 and 17, and those from 2 and 4 per cent. glucose in Pl. XXV, Figs. 19 and 20. As shown by the four figures the apex is markedly dorsiventral and curved upwards so that the tetrahedral apical cell is directed towards the dorsal side of the rhizome. It may also be seen that, although the young leaves arise in close proximity to the apical cell, they do not enclose it completely, protection being afforded by the numerous uniseriate hairs which provide a close covering.

It is apparent from the figure that the apices from 1 and 2 per cent. glucose are closely similar and differ considerably from the apices from 4 and 5 per cent. glucose. In the first place, the general form of the two groups of apices is different, those from the two lower sugar concentrations enlarge gradually until the final width of the rhizome is attained, whereas the apices from the higher sugar concentrations are more obtuse and quickly expand to the permanent dimensions. The curvature of the apex is also less marked in the higher sugar concentrations. In the two lower concentrations the curvature is such that the apical cell is directed obliquely backwards towards the base of the rhizome, but in the two higher sugar concentrations the apical cell is not quite perpendicular to the longitudinal axis of the rhizome and is directed obliquely forwards.

These differences in general form are a consequence of changes in the duration and direction of cell-division rather than of differences in the form of the constituent cells. There is no evidence of any changes in the pattern of segmentation of the apical cell and its immediate derivatives, which is very regular as already described in detail by Schneider (1913). It is the subsequent divisions in the sub-apical region which account for the differences between the two groups of apices.

In the two higher sugar concentrations active cell-division ceases at a relatively short distance (approximately 260μ) from the apical cell, when the cells assume the characteristics of the mature tissues. In the two lower sugar concentrations the actively dividing region extends for a much greater distance (approximately 800μ). The number of tangential divisions in the two types of apex remains approximately the same. It is the increase in the number of transverse divisions, and a corresponding extension of the tangential divisions over a greater longitudinal distance, which accounts for the tapering elongated apex of the plants from the lower sugar concentrations. The longitudinal extension of the growing region probably also accounts for the greater curvature of the apex in the lower sugar concentrations. This curvature evidently results from more active division and earlier vacuolation of the cells of the lower side of the rhizome, which leads to an increase in the relative length of this region. The difference between upper and lower sides is maintained for a greater distance in the lower sugar concentrations, and consequently leads automatically to an increased displacement of the apical cell.

Another striking difference between the two groups of apices lies in the degree of development of the air-space tissue. In both groups the large cortical air-spaces originate by a separation of certain cells, beginning at a distance of approximately 200μ from the apex. The cells adjoining the small schizogenously produced air-spaces then divide transversely and tangentially to form the characteristic longitudinal partitions. In the apices from the two lower sugar concentrations, as a result of the preponderance of transverse divisions in the cells adjoining the air-spaces, there is a great extension of these spaces in a longitudinal direction. The early stages of this process are well seen in tangential longitudinal sections through the apex (Pl. XXV, Fig. 18). Median longitudinal sections show that tangential division also is still active at a considerable distance from the apex so that both transverse and longitudinal partitions become considerably extended in a radial direction (Pl. XXIV, Fig. 16; Pl. XXV, Fig. 19). In the apices from the two higher glucose concentrations, on the contrary, the cells of the growing-point become mature in a region only shortly beyond the point of origin of the air-spaces and as a result only few divisions are able to occur in the cells adjoining the latter. Thus, in the mature parts of the plants from high sugar concentrations, the air-spaces are very short in both radial and longitudinal directions, and as described above the radial partitions show only 2 or 3 cells in the transverse section.

The two groups of apices also show differences between their prevascular tissue; in the apices from the higher sugar concentrations this tissue is more sharply defined and, furthermore, as a consequence of an increase in the number of longitudinal divisions, is of greater thickness than in the apices from the lower sugar concentrations.

In both sets of apices, roots and leaves arise quite normally in close proximity to the apical cell. The early segmentation of these organs is also normal and the characteristic differences between the mature organs must be referred to developments in the sub-apical regions of these organs.

DISCUSSION

The above account has shown that there are many anatomical distinctions between sporelings grown in low and high sugar concentrations. These anatomical differences run parallel with the differences in external morphology described previously (Allsopp, 1953*a*). The features, both anatomical and morphological, of the sporelings from the lower sugar concentrations (1 and 2 per cent. glucose) are those characteristic of submerged water plants, or of the water forms of amphibious plants, while the features of the sporelings from the higher sugar concentrations (4 and 5 per cent. glucose) are those characteristic of the land forms of amphibious plants. The differences in structure are sharp so that intermediate concentrations of sugar do not yield intermediate types of structure. Instead there appears to be a critical concentration of sugar above which the normal land form is maintained, and below which there is an abrupt change to the typical construction of the water form.

The principal differences between the land and water forms of a wide range of aquatic and amphibious plants were described at an early date, particularly in the thorough accounts of Constantin (1884, 1886) and Schenck (1885, 1886). This and other work has been summarized more recently by Arber (1920). Since the review by Arber many other publications dealing with this subject have appeared, but there is still no agreement as to the factors responsible for the striking dimorphism of amphibious plants.

It has been a widely held view that the characteristic morphological and anatomical features of water forms are a consequence of restricted nutrition. Thus, Goebel (1908, 1928) considered that a relative deficiency of assimilates leads to the retention of the juvenile condition, so that water forms arise, while Küster (1916) maintained that the characteristic features of water plants are examples of hypoplasy, in which complete development and differentiation of the tissues is arrested.

These views are not supported by the present work. In previous sections of the investigation (Allsopp, 1953, 1953*a*) it was shown that nutrition has a marked effect on the heteroblastic development of *Marsilea*, particularly on the degree of segmentation of the lamina, but there is no evidence that the differences between 'land' and 'water' forms from the high and low sugar concentrations are a result of any carbohydrate deficiency. Growth of the 'water' forms is very active, and furthermore the tissues of both types of sporelings contain numerous starch grains.

Apart from these considerations, anatomical observation alone demonstrates that the water form is by no means merely a reduced form arising by inhibition of the development of the more complete land form. Thus, although certain tissues such as the mesophyll are less developed in the water form, others such as the partitions between the cortical lacunae of leaf, root, and rhizome are more developed than in the land form, and this is true for naturally occurring plants as well as for those growing in artificial media. Examination of the rhizome apices has shown that the differences between the two types of sporeling are largely due to changes in the duration and direction of cell division in the sub-apical region, a region whose importance has already been stressed by Wardlaw (1952).

It is evident therefore that the distinctive features of the water form are not a consequence of restricted carbohydrate nutrition. If we reject this nutritional explanation we are left with the alternative possibility that changes in sugar concentration, when all other conditions remain constant, exert their effects as a result of alteration of the osmotic properties of the medium. It is considered that the increased osmotic pressures of the higher sugar concentrations are analogous in their effects on the plant to growth under normal land conditions. On this view the formation of land or water forms is a direct response to the water relations of the environment, and not an indirect response consequent on nutritional inadequacy. Evidence of this was advanced in 1902 by McCallum, who considered that the water form of *Proserpinaca palustris* arose as a result of suppression of transpiration in the aqueous medium.

SUMMARY

Sporelings of *Marsilea*, grown aseptically in various concentrations of sugars, particularly glucose, have been investigated anatomically.

Corresponding to the features of the external morphology, the anatomy of the sporelings from the low sugar concentrations (1 and 2 per cent. glucose) has many of the characteristics of the water forms of amphibious plants, while that of the sporelings from the higher sugar concentrations (4 and 5 per cent. glucose) has the characteristics of typical land forms.

It is shown that the 'water' forms are not merely reduced forms arising by inhibition of the development of a more complete land form. In particular, the direction and duration of cell-division are different in the rhizome apices of the two types of sporeling.

The view is advanced that the differences between land and water forms are not a result of changes in carbohydrate nutrition, but, in the experimental conditions described, arise in response to differences in the osmotic concentration of the culture medium, which are analogous to the differences in availability of water in normal aquatic and terrestrial conditions.

ACKNOWLEDGEMENTS

I wish to thank Professor C. W. Wardlaw for his helpful advice and friendly criticism, and Mr. E. Ashby for the photographs reproduced in the Plates.

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EXPLANATION OF PLATES

Illustrating A. Allsopp's article on 'Experimental and Analytical Studies of Pteridophytes. XXIV'.

PLATE XXIII. *Marsilea Drummondii*

Figs. 1, 2, 3, and 4. Transverse sections of laminae of leaves of sporelings from media containing 5, 1, 4, and 2% glucose respectively. (×200.)

Figs. 5, 6, 7, and 8. Transverse sections of petioles of sporelings from media containing 5, 1, 4, and 2% glucose respectively. (×75.)

Figs. 9, 10, 11, and 12. Transverse sections of roots of sporelings from media containing 5, 1, 4, and 2% glucose respectively. (×100.)

PLATE XXIV. *Marsilea Drummondii*

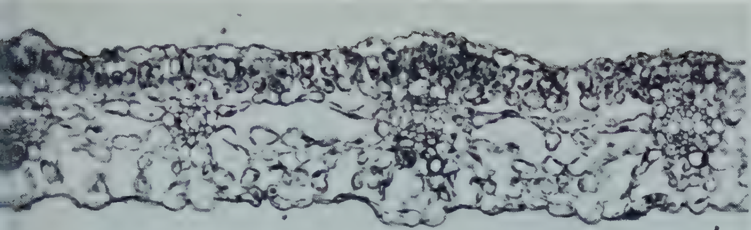
Figs. 13, 14, and 15. Transverse sections of rhizomes from media containing 1, 2, and 4% glucose respectively. (×75.)

Figs. 16 and 17. Median longitudinal sections through rhizome apices from media containing 1 and 5% glucose respectively. (×150.)

PLATE XXV. *Marsilea Drummondii*

Fig. 18. Tangential longitudinal section of rhizome apex from 1% glucose whose median longitudinal section is illustrated in Fig. 16. Fig. 18 shows origin of air-spaces of cortex. The apical cell of a lateral bud in shown, with a root primordium below. The projection above is the base of a young leaf.

Figs. 19 and 20. Median longitudinal sections of rhizome apices from 2 and 4% glucose respectively. Both sections show the origin of root primordia on the lower side of the apex. (×150.)



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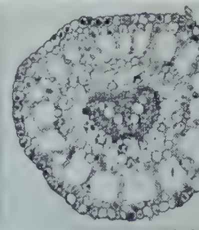

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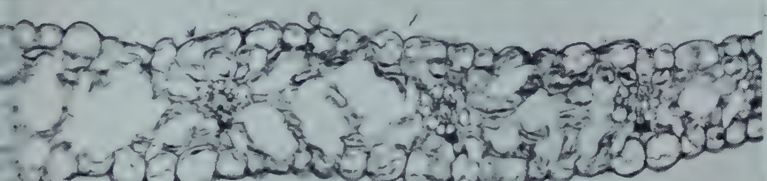

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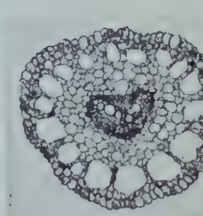

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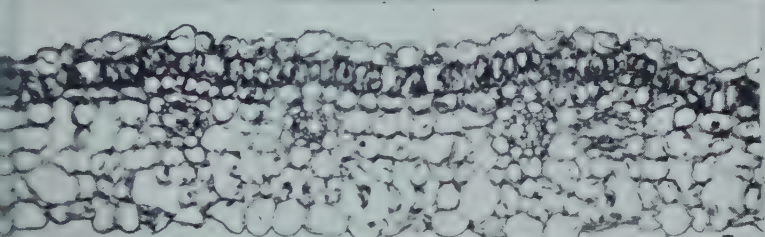

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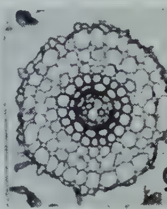

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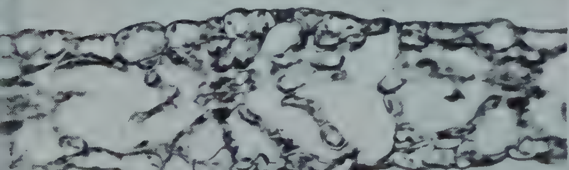

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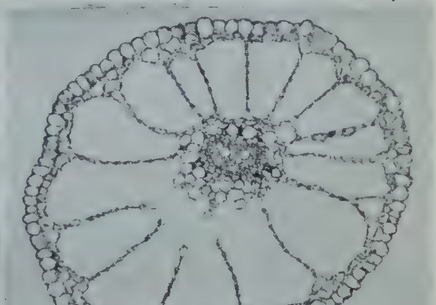

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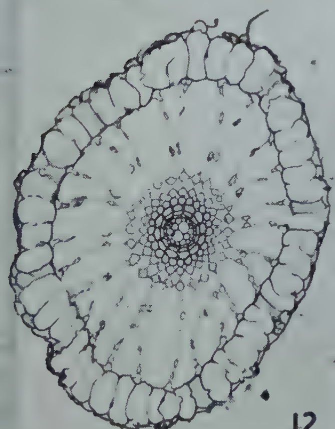

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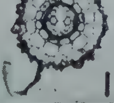

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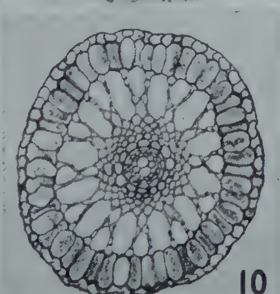

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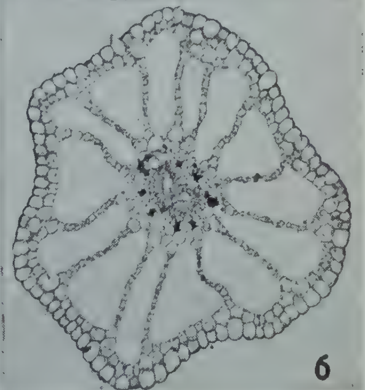

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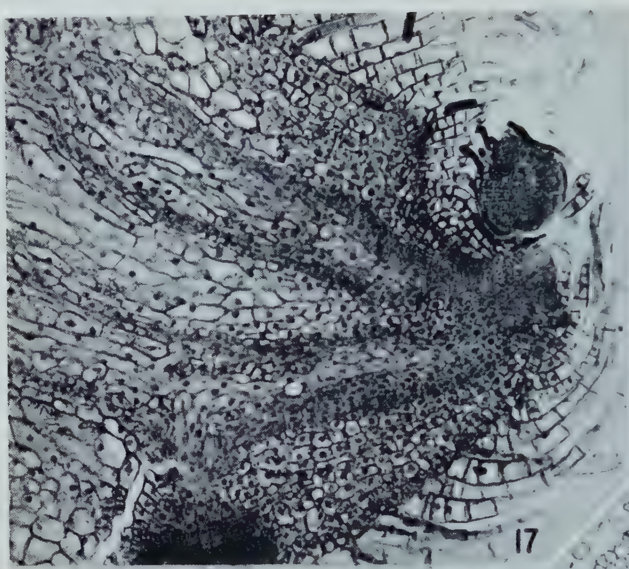

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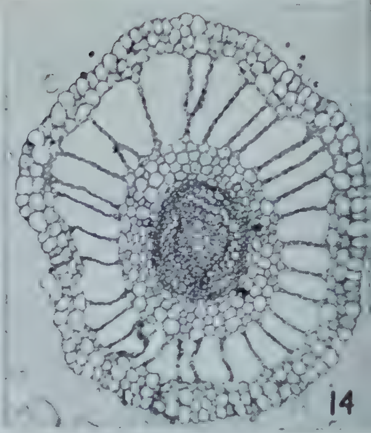

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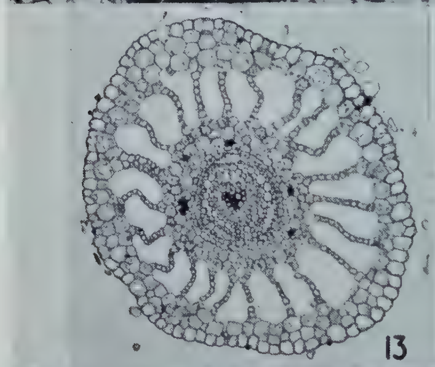

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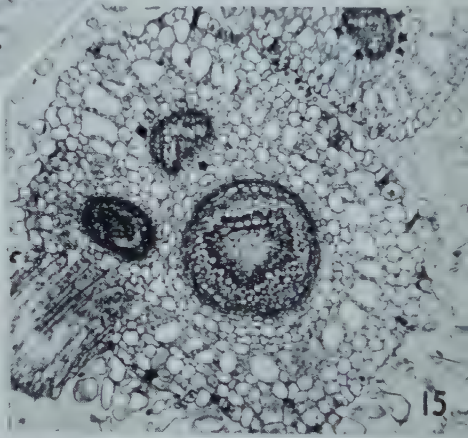

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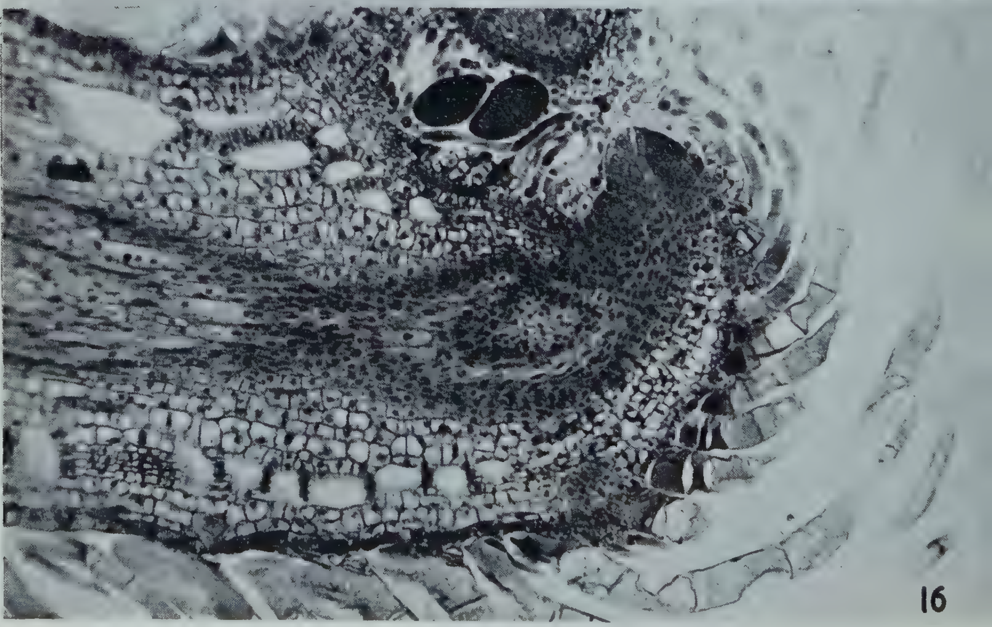

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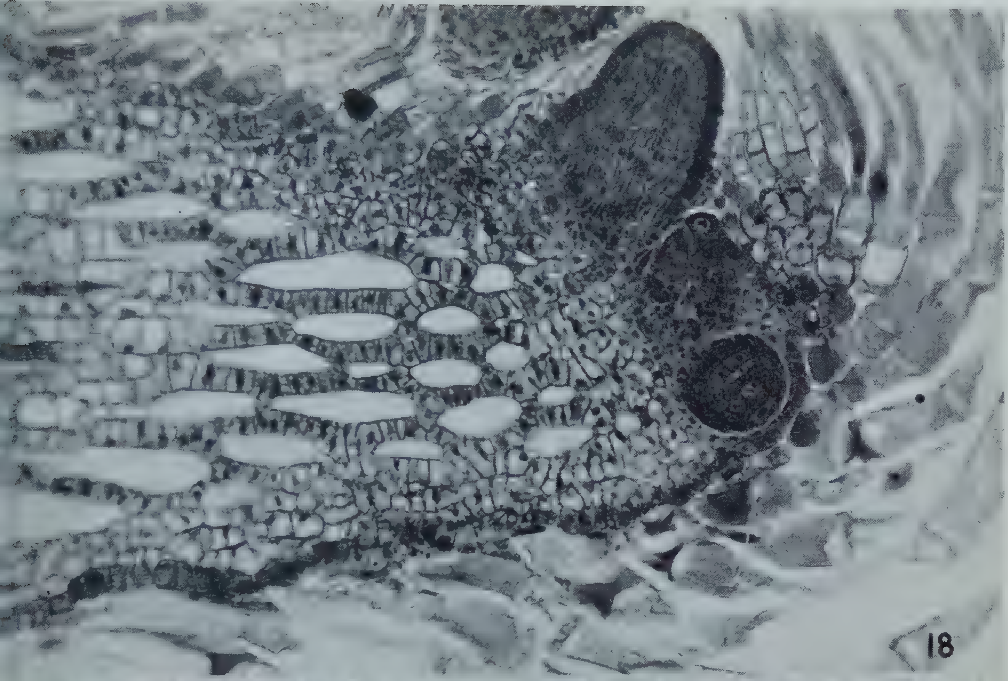

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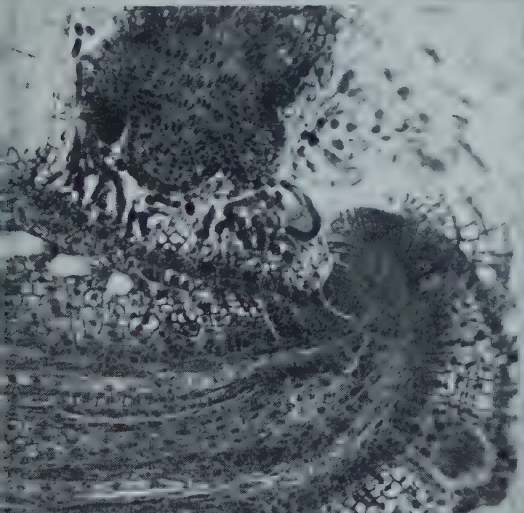

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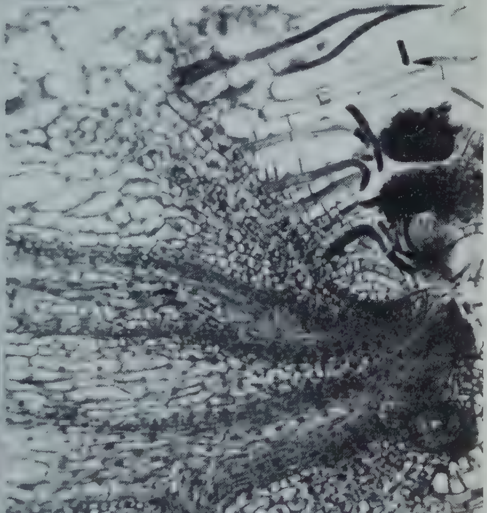

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A. ALLSOPP

The Movement of Chloramphenicol and Streptomycin in Broad Bean and Tomato Plants

BY

D. PRAMER[1]

(*Imperial Chemical Industries Butterwick Research Laboratories, Welwyn, Herts.*)

With two figures in the Text

ABSTRACT

The concentration of chloramphenicol in treated plants varied directly with that in solution over a range of 10–500μg./ml. Cut shoots contained more antibiotic than rooted plants. Chloramphenicol was distributed throughout plants treated with 200μg./ml. solutions for 19 hours, but the concentration at the base of plants was greater than that at the top. When treatment was continued for 5 days the concentration of chloramphenicol was uniform throughout the plant. If, following treatment, the plants were grown in water for 5 days, top leaves contained more antibiotic than lower leaves. The accumulation of chloramphenicol in rooted broad bean plants was a linear function of water uptake.

Rooted broad bean plants grown for 18 hours in streptomycin solutions containing 500μg./ml. had a trace of antibiotic in bottom leaves only. No streptomycin was present in plants treated with less concentrated antibiotic solutions. Streptomycin moved more readily in cut shoots of broad bean and cut shoots and rooted tomato plants, but the antibiotic content of bottom leaves was much greater than that of top leaves. The accumulation of streptomycin in rooted broad bean plants was exceedingly slow.

INTRODUCTION

A RECENT review of the literature (Crowdy and Pramer, 1954*a*) has shown streptomycin to be absorbed and translocated by all but one of thirteen plant species tested. The exception, broad bean (*Vicia faba* L.), was reported in preliminary studies by Crowdy and Pramer (1954*b*). Though streptomycin was demonstrated to move in cut shoots of broad bean, no antibiotic was detected in leaves of root-treated plants. Chloramphenicol was absorbed and translocated by all plant species tested, including the broad bean.

The present communication describes further studies of the uptake and translocation of chloramphenicol and streptomycin by broad bean plants. Comparative data obtained from studies with tomato (*Lycopersicum esculentum* Mill.) are also presented.

MATERIALS AND METHODS

Seedlings were raised in sand culture and transferred to beakers containing 250 ml. of nutrient solution; when plants were ready for treatment the

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[1] Present address: Dept. of Microbiology, New Jersey Agric. Expt. Station, Rutgers University, New Jersey, U.S.A.

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nutrient was replaced by a solution of antibiotic. Experiments were conducted in a chamber at 25° C. and a relative humidity of about 60 per cent. Mercury-vapour lamps provided 14 hours of daylight. Individual beakers containing two plants and solution were weighed at the beginning and end of the treatment period. The difference between these weights provided a measure of water uptake by the plants.

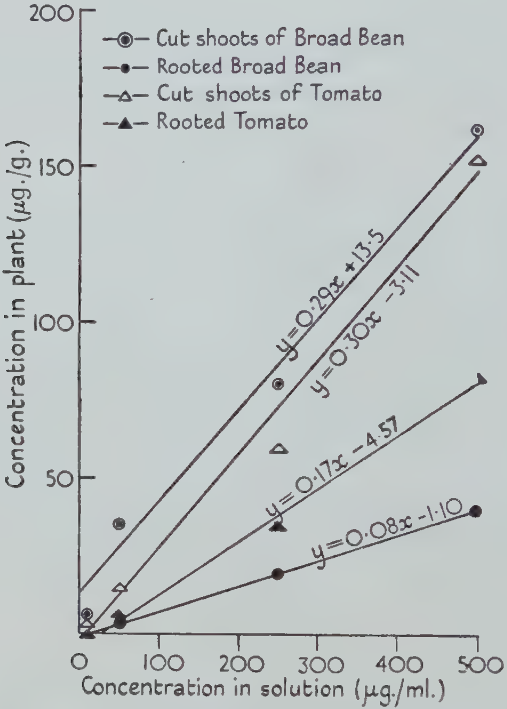

FIG. 1. Relationship between the concentration of chloramphenicol in solution used for treatment and the concentration in the plants.

The antibiotic concentration in treated plants was measured by assaying sap expressed from tissue killed by immersion for 30 seconds in ether. Pressures of approximately 300 lb. per sq. in. were used. Ether present in the collected sap was reduced to a non-toxic level by evaporation under reduced pressure at room temperature. Plant saps thus obtained were diluted with distilled water and the antibiotic content assayed by the cylinder-plate technique. The assay medium was Lab-Lemco agar supplemented with 0·3 per cent. yeast extract and seeded with a *Bacillus subtilis* (N.C.T.C. 7241) spore suspension. At least two, and usually three, dilutions of each sample were assayed. A detailed description of these methods has been presented previously (Crowdy and Pramer, 1954*b*).

EXPERIMENTAL PROCEDURES AND RESULTS

1. *Relationship between the concentration of antibiotic in solution and the concentration in plants*

Cut shoots and rooted broad bean and tomato plants were treated with various concentrations of chloramphenicol and streptomycin for 18 hours in the experimental chamber. After treatment roots and bases of cut shoots that had been immersed in antibiotic solution were discarded. Expressed sap collected from the stems and leaves was assayed. The two plants in each beaker were combined before pressing and duplicate determinations were made for each concentration of antibiotic studied. The experiment was repeated at various times. Figs. 1 and 2 illustrate the results obtained.

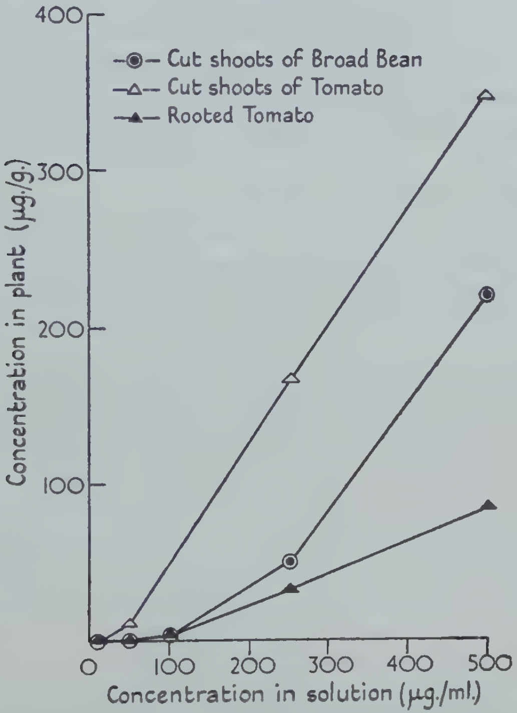

FIG. 2. Relationship between the concentration of streptomycin in the solution used for treatment and the concentration in the plants.

In these trials water uptake did not vary significantly from a mean of 0·8 ml./g. of plant.

Fig. 1 shows that over a range of 10–500 μg./ml. there is a linear relationship between the concentration of chloramphenicol in solution and the concentration in rooted broad bean and tomato plants. Cut shoots were found to present a similar picture; however, at any given concentration of antibiotic in solution, the concentration found in cut shoots was always greater than that in rooted plants.

A trace of streptomycin was detected in the bottom leaves only of rooted broad bean plants treated with solutions containing 500 µg./ml. This small quantity was diluted beyond detection when the whole plant was treated as a unit for assay. No streptomycin was found in the tops of rooted bean plants treated with less concentrated antibiotic solutions. Streptomycin was translocated in cut shoots of broad bean and in cut shoots and rooted tomato plants (Fig. 2). It appears that a relatively large amount of streptomycin must be supplied to the base of plants before any can be detected in the stems and leaves. Once this requirement is satisfied the relationship between the streptomycin concentration in solution and that in the plant approaches linearity. This behaviour differs from that recorded for chloramphenicol.

2. *The distribution of antibiotics in plants*

Distribution studies were made by treating cut shoots and rooted broad bean and tomato plants for 18 hours with solutions containing 200 µg./ml. of chloramphenicol or streptomycin. After treatment, individual plants were cut into three sections: 1, top; 2, middle; 3, bottom. Roots and the base of cut shoots that had been immersed in antibiotic solutions were discarded. The antibiotic concentrations present at different levels in treated plants as determined by the assay of expressed sap are shown in Table I.

TABLE I

The Distribution of Antibiotics in Plants

Plant	Concentration of antibiotic (µg./g. fresh wt.)					
	Chloramphenicol			Streptomycin		
	1.	2.	3.	1.	2.	3.
Broad bean:						
cut shoot	40	41	65	0	7	122
rooted plant	17	29	27	0	0	0
Tomato:						
cut shoot	51	62	70	2	55	240
rooted plant	10	29	42	5	20	40

1, 2, and 3 represent the top, middle, and bottom of plants.

Chloramphenicol was distributed throughout treated plants, but in every case lower sections contained more antibiotic than upper sections. No streptomycin was demonstrated in rooted broad bean plants. Cut shoots of broad bean showed a relatively high streptomycin concentration in bottom sections, but no antibiotic was present in top sections. Streptomycin moved more readily in tomato plants. The antibiotic was detected at all levels, but the concentration in bottom sections was 8–10 times greater than that in top sections. In all studies the concentration of antibiotic in cut shoots was greater than that in rooted plants.

3. *The accumulation of antibiotics in broad bean plants*

Observations on the accumulation of chloramphenicol and streptomycin in rooted broad bean plants are presented in Tables II and III. Plants were grown in antibiotic solutions containing 200 μg./ml. and removed for assay at various times after the start of treatment. The concentration of antibiotic in each of three levels of the shoot was determined. Roots were discarded. Duplicate determinations were made at each time interval and water uptake was measured. During the period of the trial the average increase in concentration (y) of chloramphenicol in the plants was shown, by the method of least squares, to be a linear function of the water uptake (x) fitted by the following regression line.

TABLE II

The Accumulation of Chloramphenicol in Broad Bean Plants

	Concentration of antibiotic (μg./g. fresh wt.)			
	Time (hrs.)			
Level in plant	17.	41.	65.	137.
1	8	12	25	36
2	15	21	30	38
3	14	17	26	37
Water uptake (ml./g.)	0·9	1·6	2·5	6·0

$$y = 3{\cdot}20x + 15{\cdot}04.$$

The data in Table II indicate a slow distribution of chloramphenicol within plants. After 17 hours the bottom of plants contained more antibiotic than the top, but this gradient disappeared after 137 hours.

TABLE III

The Accumulation of Streptomycin in Broad Bean Plants

	Concentration of antibiotic (μg./g. fresh wt.)				
	Time (hrs.)				
Level in plant	19.	67.	91.	137.	232.*
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	2	3	13
Water uptake (ml./g.)	0·9	2·2	4·5	6·6	—

* Leaves flaccid, plants wilting.

Streptomycin could not be detected in plants grown in solutions containing 200 μg./ml. for 67 hours (Table III). Bottom leaves were found to contain only 2 μg./g. after 91 hours, and though the concentration increased to 13 μg./g. at 232 hours, it remained localized in bottom leaves.

4. *The redistribution of chloramphenicol in broad bean plants*

Plants were treated for 19 hours in a solution containing 200μg./ml. of chloramphenicol and then transferred to solutions without antibiotic. The chloramphenicol concentration at different levels in the plants was determined at the termination of treatment and periodically thereafter.

TABLE IV

The Redistribution of Chloramphenicol in Broad Bean Plants

	Concentration of antibiotic (μg./g. fresh wt.)			
	Time after treatment (hrs.)			
Level in plant	0.	24	48.	72.
1	8	19	17	28
2	21	27	30	18
3	22	23	23	13

Table IV shows that following the initial treatment period a concentration gradient was present in the plants; the concentration of chloramphenicol in the bottom of plants was approximately 3 times that at the top. After 72 hours this gradient was reversed and the antibiotic content at the top of plants was more than twice that at the bottom. At intermediate times (24–48 hours) the highest concentration of antibiotic appeared in the centre portion of plants.

Absorbed chloramphenicol appeared stable. Plants contained a mean concentration of 17μg./g. immediately following treatment and this value increased to 23μg./g. after 24 hours. The increase would result from movement into shoots of chloramphenicol present in roots when treatment was terminated.

DISCUSSION AND SUMMARY

Chloramphenicol. The present studies have shown chloramphenicol to be absorbed through the roots of broad bean and tomato plants and translocated to the leaves. The absorption of chloramphenicol by higher plants has been previously reported by Pramer (1953) and Crowdy and Pramer (1954*b*).

The concentration of antibiotic in treated plants varied directly with that in solution over a range of 10–500μg./ml. At any given concentration in solution, cut shoots were found to contain more antibiotic than rooted plants. Roots appear to limit the amount of antibiotic that enters a plant. This discriminatory ability of roots has been previously demonstrated by a failure to detect various antibiotics in stem-exudates from decapitated cucumber seedlings (Pramer, 1953).

Chloramphenicol was found to be distributed throughout plants treated for 19 hours, but the concentration at the base of plants was greater than at the top. When antibiotic treatment was continued for 5 days this gradient tended to disappear and the concentration of chloramphenicol became uniform throughout the plant. If, following 19 hours of treatment, plants were grown in water for 3 days, the gradient was reversed; top leaves had a greater antibiotic content than bottom leaves. These results suggest a relatively slow movement of chloramphenicol up the plant (Table IV).

Chloramphenicol did not appear to accumulate in cucumber seedlings (Pramer, 1953), but present studies demonstrated accumulation in broad beans. The concentration of chloramphenicol in beans was found to be a linear function of water uptake.

The recovery of chloramphenicol from tissue of treated broad beans (Crowdy, Gardner, Grove, and Pramer, 1954) provided evidence that the compound is absorbed and translocated without alteration, and, therefore, is responsible for the antibiotic activity measured in treated plants.

Streptomycin. A trace of streptomycin was detected in the bottom leaves only of rooted broad bean plants grown in solutions containing 500μg./ml. for 18 hours. No streptomycin was present in plants treated with less concentrated antibiotic solutions. Streptomycin moved more readily in tomato and was detected throughout plants after 18 hours; however, the concentration in bottom leaves was 8 times that in top leaves.

It appears that a high concentration of streptomycin must be applied to the base of plants before any is detected in aerial portions. When this requirement was satisfied the relationship between the concentration in solution and that in plants tended to be linear. Cut shoots were found to contain more antibiotic than rooted plants when both were similarly treated.

In accumulation studies the movement of streptomycin was exceedingly slow. No antibiotic was detected in plants grown for 67 hours in solutions containing 200μg./ml. Bottom leaves of plants treated for 91 hours had an antibiotic content of only 2μg./g. This concentration increased to 13μg./g. after 232 hours, but the antibiotic remained restricted to bottom leaves. Though streptomycin has been reported to move more slowly than penicillin (Krasilnikov, 1952; Charles, 1953), the low rate of movement noted in rooted broad bean plants has not been previously recorded. The work of Mitchell et al. (1954) with dwarf French beans is not comparable since the antibiotic was applied to stems and not through roots. Present studies demonstrated a more rapid movement of streptomycin in cut shoots than in rooted plants.

Charles (1953) has suggested that xylem elements are negatively charged. This would interfere with translocation of positively charged streptomycin molecules but not hinder movement of a neutral substance such as chloramphenicol. This supposition would serve to explain the observed differential rate of movement of the two antibiotics in a single plant species. However, to interpret the more rapid movement of streptomycin in tomato than in broad bean it is necessary to conclude the vessels of the former plant species are more negatively charged than those of the latter. Whether or not this condition does prevail remains for future investigation.

ACKNOWLEDGEMENTS

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Anthocyanins in Bananas

BY

N. W. SIMMONDS

(*Banana Research Scheme, Imperial College of Tropical Agriculture, Trinidad, B.W.I.*)

With one Figure in the Text

ABSTRACT

The bracts of wild banana species are pigmented by glycosides (probably 3-diglucosides) of four combinations of anthocyanidins: cyanidin-pelargonidin; cyanidin-delphinidin; delphinidin-petunidin-cyanidin-malvidin-peonidin; malvidin-peonidin. The proportions of the various components are slightly variable as between clones of one species and even between samples, but the general pattern is characteristic of a species and is therefore of considerable taxonomic value. In *Musa acuminata* a cline in bract colour is related to variation in oxidation and methylation of the anthocyanidins. Yellowness of (i.e. absence of anthocyanin in) the bracts of a strain of the same species is probably caused by a single recessive gene. The bracts of edible bananas have, broadly, the pigments expected on taxonomic and genetic grounds but show an (unexplained) tendency towards a generally lower level of methylation of the anthocyanidins. Leucoanthocyanins are present in most parts of the banana plant; they yield delphinidin and cyanidin in proportions that vary approximately with overall intensity. In discussion, the taxonomic interest of the results and the technically favourable nature of bananas for investigation of the leucoanthocyanins and the biogenesis of the visible pigments are emphasized.

INTRODUCTION

TWO anthocyanins and one anthocyanidin derived from a leucoanthocyanin have previously been reported in the genus *Musa*. A cyanidin 3-pentose-glucoside occurred in the bracts of *Musa basjoo* and pelargonidin 3-monoside in the fruits of *Musa coccinea* (Lawrence, Price, Robinson, and Robinson, 1939); Robinson (1937) identified delphinidin in the products of hot acid treatment of the fruit pulp of an edible variety.

The work reported here was undertaken when the chromatographic methods of Bate-Smith (1949) became available for the separation and identification of the anthocyanins and their aglycones; it has a taxonomic and genetic bias.

Thanks are due to Dr. E. C. Bate-Smith for information on unpublished techniques, and to Dr. W. G. C. Forsyth and Mr. K. De Witt for much help in various ways.

MATERIALS AND METHODS

The collections of cultivated and wild bananas at the Imperial College of Tropical Agriculture were used. Most work has been done on the conspicuously coloured bracts that subtend the male flowers, but some attention has

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also been paid to fruit and plant colours. Banana flowers themselves are mostly white or yellowish.

The pigments were usually extracted in cold 1 per cent. (by volume) aqueous HCl, occasionally in 1 per cent. methanolic HCl. The glycosides were separated chromatographically on Whatman No. 1 paper in the butanol-acetic acid-water solvent (40:10:50) recommended by Bate-Smith (1949; Bate-Smith and Westall, 1950). Hydrolysis of the anthocyanins was carried out by heating the pigments in 5N. aqueous HCl on the water-bath for 20 minutes. The aglycones were concentrated into amyl alcohol and examined chromatographically in butanol-2N.HCl (Bate-Smith, 1949) or in the Forestal solvent (60 acetic acid: 20 water: 6HCl; Bate-Smith, 1954). The latter was in general more useful because it gave good separation without tailing. Complex mixtures were resolved by two-way chromatography, Forestal first and butanol-2N.HCl second; this procedure was particularly useful for separating peonidin from malvidin and traces of petunidin from cyanidin.

Most of the work was carried out at 20° C. and no difficulty was experienced in obtaining consistent and repeatable R_F values for the anthocyanidins in the Forestal solvent. Values for butanol-2N.HCl and for glycosides in butanol-acetic acid, however, were rather variable; since chemically known standards were not available, reliable R_F values for the glycosides cannot be reported here. The anthocyanidin responsible for each anthocyanin spot was recognized by (*a*) its colour; (*b*) reaction on the paper to a drop of dilute ferric-chloride; and (*c*) by cutting up papers, eluting, hydrolysing, and identifying the aglycone chromatographically.

Leucoanthocyanidins were studied by heating the material to be tested in 3-5N.HCl, decanting or filtering, extracting, with amyl alcohol and examining chromatographically.

RESULTS

The anthocyanidins

Six anthocyanidins have been recognized in bananas, thus:

		R_F values*		
Anthocyanidin.	Colour.	Forestal.	Bu-2N.HCl.	$FeCl_3$.
Delphinidin	Blue-red	0·30	0·35	+
Petunidin		0·46	(0·47)	+
Cyanidin	ranging	0·50	0·69	+
Malvidin	to	0·60	0·53	−
Peonidin		0·63	0·72	−
Pelargonidin	orange-red	0·68	0·80	−

* From Bate-Smith (1949, 1954; bracketed value for petunidin determined in this work as average of 10 runs, range 0·44–0·49). The ferric chloride reaction, it may be noted, is satisfactory if the spot is strong, but the characteristic fading blue margin with pale centre is difficult to discern if the spot is weak.

The anthocyanins

By a combination of the tests described above it appeared that each anthocyanidin was represented by a single glycoside and that, most probably, a single glycosidal type was common to all. A possible exception to this statement is furnished by *M. balbisiana* in which two cyanidin glycosides sometimes appeared to be present (Fig. 1). Mass chromatography on large sheets of paper of an extract of *Musa acuminata* clone Long Tavoy (a complex mixture, see below) yielded paper strips containing the anthocyanins uncontaminated by any other detectable phenols. Elution and hydrolysis yielded glucose as the only detectable sugar. Similarly, glucose only was found in the products of hydrolysis of a picrate obtained from *Musa balbisiana*.[1]

The distribution number in the test-tube of the anthocyanins between 1 per cent. HCl and amyl alcohol is low; it remains low on dilution with 1 per cent. HCl but rises somewhat on saturation with salt. These facts, coupled with the observation that R_F values in butanol-acetic acid are low, suggest that the anthocyanins are biosides, probably 3-diglucosides. It should be added that distributions in the test-tube were unchanged by mild alkaline or acid hydrolysis; acylation therefore probably does not occur.

The anthocyanins of banana bracts are not evidently co-pigmented, as shown by absence of colour change on boiling or on extraction of anthoxanthins with ethyl acetate.

Occurrence in the bracts of wild bananas and certain hybrids

The following list summarizes the occurrence of the anthocyanins in the bracts of ten wild banana species. In brackets after each specific name are given the basic chromosome number ($n = 10$ or 11), the section of the genus to which it belongs and the number of distinct clones examined. The four main patterns of anthocyanin spots are shown in Fig. 1.

A. *Unmethylated pigments*

1. Pelargonidin-cyanidin mixture, the former predominant: *M. coccinea* (10, Callimusa, 1 clone, Fig. 1A).
2. Cyanidin-delphinidin mixtures, the former predominant: *M. balbisiana* (11, Eumusa, 10 clones, Fig. 1B); *M. velutina* (11, Rhodochlamys, 1 clone); *M. laterita* (11, Rhodochlamys, 1 clone—delphinidin very faint, sometimes not detected); *M. lolodensis* (10, Australimusa, 1 clone—pigmentation very faint and the occurrence of traces of methylated pigments, though not detected, is not excluded as a possibility).

B. *Partially methylated mixtures*

Cyanidin-delphinidin mixtures (i.e. various combinations of delphinidin, petunidin, malvidin, cyanidin, and peonidin): *M. acuminata* (11, Eumusa, many clones—see below and Fig. 1C); *M. textilis* (10, Australimusa, 2 clones—small amounts of methylated pigments only, not detected in some runs).

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[1] I am much indebted to Dr. W. G. C. Forsyth for carrying out these analyses.

C. *Completely methylated mixtures*

> Cyanidin-delphinidin mixtures (i.e. combinations of peonidin and malvidin, one component sometimes very faint or apparently absent): *Musa ornata* (11, Rhodochlamys, 1 clone); *Musa violascens* (10, Callimusa, 1 clone); 'Mariani' (an undescribed species from Assam, 11, Eumusa, 2 clones, Fig. 1D).

Variation in bract colour in bananas is therefore procured essentially by variation in the level of oxidation and degree of methylation of the basic anthocyanidin skeleton. Both features—oxidation and methylation—though somewhat variable within a species, are broadly characteristic of the species and are therefore taxonomic characters of considerable value.

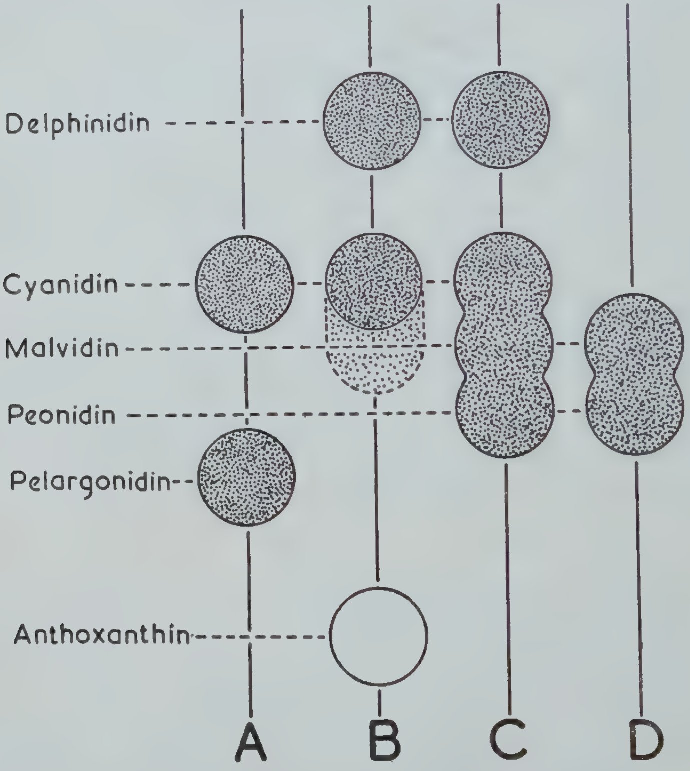

FIG. 1. Diagram to show the four main patterns of anthocyanin spots in butanol-acetic acid. A, *Musa coccinea*; B, *M. balbisiana*; C, *M. acuminata*; D, 'Mariani'. The position of the petunidin glycoside in *M. acuminata* (when present) has not been determined with certainty, but probably it lies as a faint trail between the cyanidin and delphinidin glycosides. The faster cyanidin component of *M. balbisiana* (shown in dotted outline) is inconstantly present.

Special interest centres on *Musa acuminata* as an extremely variable species well represented in the I.C.T.A. collections and of profound economic importance as the ancestor, direct or indirect, of almost all the cultivated bananas.

Two-way chromatograms of the bract anthocyanidins of a number of clones were run and the whole series repeated. Each paper was examined by two independent observers and the relative intensities of the various pigments scored visually on a percentage basis. Statistical analysis showed that agreement between observers was good and that there were undoubtedly real differences between clones (Simmonds, 1954*a*). The leucoanthocyanidins of the mature bracts (which could falsify the results) were not normally extracted by the cold 1 per cent. HCl used for extracting the anthocyanins (see below). Nevertheless a watch was kept for anomalously high delphinidin frequencies as judged by comparison with the corresponding delphinidin-anthocyanin spot; three samples in which some leucoanthocyanidins had apparently been extracted were rejected. Table I summarizes the results.

TABLE I

Anthocyanidins in Musa acuminata

			%[1]					%[1]	
Clone.	Origin.	Degrees Latitude.	Dp.	Pt.	Cy.	Mv.	Pn.	Dp.	Meth.
E	Java	−7	15	8	51	14	12	37	34
Buitenzorg	,,	−7	11	0	31	10	48	21	58
Makassar	Ceram	−3	24	5	42	10	19	39	34
Borneo 2	Borneo	0	10	0	28	11	51	21	62
Sarawak	,,	0	9	0	29	16	46	25	64
Selangor	Malaya	+5	8	5	24	43	21	56	69
Langet A	,,	+5	5	0	23	22	51	27	73
Pahang	,,	+5	13	6	40	26	15	45	47
Annam	Indo-China	+12	20	10	11	49	10	79	69
Calcutta 4	Burma	+15	5	5	10	73	8	83	86
Long Tavoy	,,	+15	13	9	15	46	18	68	73
Pemba	Zanzibar	—	0	0	36	15	49	15	64

[1] Dp, delphinidin; Pt, petunidin; Cy, cyanidin; Mv, malvidin; Pn, peonidin.
[2] Dp, total delphinidin and its derivatives; Meth, methylated pigments.

Bract colour varies geographically from bluish-red in the south through red in the centre of the range (Malaya) to reddish-blue in the north. Thus there is a cline in bract colour in *Musa acuminata* which appears to have as its chemical basis changes in oxidation and methylation of the anthocyanins, the former causing a blueing of colours in the northern part of the cline, the latter having a reddening effect in the southern part. This interpretation represents a slight modification of one previously put forward (Simmonds, 1954*a*).

In Table I are given data for a clone which cannot be included in the general sequence of *acuminata* types. Pemba undoubtedly belongs to *M. acuminata* but came from Pemba, Zanzibar Protectorate, not from Asia. Probably it is a naturalized alien introduced to Africa by human travellers (Simmons and Shepherd, 1951). Phenotypically it resembles the Malayan form of the species and its pigment chemistry at least does not contradict this diagnosis (cf. data for Langet A, for example).

A number of hybrids (mainly interspecific) having origins from clones that bore various mixtures of cyanidin and delphinidin variously methylated were also examined. The results were (0 = unmethylated; 1 = partly methylated; 2 = fully methylated):

Pigments.		Numbers of hybrids examined.
Parents.	F_1 progeny.	
0×0	0	5
0×1	1	5
0×2	(1–)2	2
1×1	1	10
1×2	1	11
2×2	2	3

The conclusions are simple: (1) crossing of like parents produces only the parental phenotype in the F_1; (2) genes for partial methylation are dominant to all others; (3) genes for full methylation are dominant to those for non-methylation but recessive to those for partial methylation. A dominance sequence may therefore be written:

$$1 > 2 > 0.$$

These results relate to seeded bananas only. An experimental hybrid (S.H. 62–1) between an edible diploid form of *Musa acuminata* (clone Pisang Lilan) (1) and seeded *Musa balbisiana* (0) which would be expected to resemble the *acuminata* parent (1) gave no evidence of the presence of methylated pigments. In this case, therefore, genes for non-methylation were dominant to those for partial methylation and it seems that this behaviour is associated in some way with the genetical and physiological background of parthenocarpy. More data on this subject will be given below.

A note on anthoxanthins may conveniently be inserted at this point. Several are present of which some at least seem to be common to more than one species. In particular *M. violascens* and *M. ornata* appeared to be identical in this respect as in their anthocyanins; since they have different basic chromosome numbers ($n = 10$ and 11 respectively) and belong to different sections of the genus, they appear to constitute a rather striking instance of parallel chemical evolution. In *Musa balbisiana* a conspicuous anthoxanthin was invariably present, moving somewhat in advance of the anthocyanins (Fig. 1). It was absent from or at most very faint in chromatograms of clones of *Musa acuminata*. The combination of unmethylated anthocyanins with this conspicuous anthoxanthin is therefore diagnostic of *M. balbisiana* as compared with *M. acuminata*. It will be shown below that this fact has considerable value for the diagnosis of origins of edible banana varieties.

Anthocyanidins in the bracts of cultivated bananas

Table II summarizes the results. An interpretation in taxonomic terms (Simmonds and Shepherd, 1954) is as follows:

Group I: Diploid *acuminata*, all having the anthocyanins of wild *M. acuminata*.

Group II: Diploid *acuminata-balbisiana* hybrid type having the anthocyanins of the *acuminata* parent but the anthoxanthin of *balbisiana*. This plant is presumed to have had the same origin as the experimental hybrid S.H. 62–1 mentioned in the previous section (Dodds and Simmonds, 1948). Evidently genes for partial methylation are dominant in some combinations of this cross but not in others.

TABLE II

Distribution of Anthocyanidins in the Bracts of Cultivated Banana Varieties

Taxonomic group.	Cyanidin-delphinidin mixtures.	
	Partly methylated.	Unmethylated.
I	Pisang Lilan (1, 3), Tongat (1), Bande (2), Palembang (1), Sucrier (1)	—
II	Ney poovan (1, 3)	—
III	Gros Michel (1), Green Red, Red (1), Oratava, Palimbang (1), Marathuva (2), Rajah (1), Rio	Cavendish group (Lacatan, Robusta, Giant Cavendish, Dwarf Cavendish)
IV	J 234 (1), J 1877 (1)	—
V	Mysore (1, 3), Pome (2, 3), Nadan (2, 3)	King (3), Plantain (3), Celat (3), Rajapuri (3, 4), Grindy (3, 4), Nendra padaththi (3, 4)
V	—	Bluggoe (3), Awak Legor (3), Ney Mannan (3), Pacha bonthus bathees (3).

Notes: (1) peonidin prominent; (2) methylation slight; (3) conspicuous *balbisiana* anthoxanthin present, inconstantly in Pisang Lilan; (4) traces of methylated pigments possibly present.

Group III: Triploid *acuminata*, all expected to have partly methylated pigments. However, four members of the Cavendish group of bananas—which unquestionably belongs here—have unmethylated pigments. This is powerful evidence that they are indeed a group of subvarieties related to each other by somatic mutation (Simmonds, 1954*b*, for review and references).

Group IV: Tetraploid *acuminata* derived from the pollination of 'Gros Michel'. They have the pigments expected.

Group V: Triploid hybrid types, predominantly *acuminata*. They would be expected in the light of results with hybrids between wild types all to have methylated pigments. In fact, the majority have unmethylated anthocyanins and all have the anthoxanthin characteristic of the *balbi-*

siana parent. The plantain is particularly interesting in this connexion, for its relationship to *M. balbisiana* has only recently been recognized; this chemical information lends support to the formal taxonomic conclusion which has significance for the identification of the type of *Musa paradisiaca* L. and hence for the botanical nomenclature of the bananas as a whole.

Group VI: Triploid hybrid types predominantly *balbisiana* in origin and having the pigments expected of them.

The general conclusions to be drawn from these results are: (1) that the expression of genes procuring methylation is weakened in the genetic milieu of edibility (compare the results from hybrids between wild types with, say, the edible group V described above; see above remarks on the Cavendish group and note 2 to Table II; (2) that, when allowance is made for this, pigment chemistry agrees well with taxonomy; and (3) identification and study of distribution of the anthoxanthin(s) concerned might weli prove taxonomically extremely informative.

The occurrence of acyanic bracts

An introduction to the I.C.T.A. collections of *Musa acuminata* from Ulu Langet, Malaya, was grown from seed and found to segregate for bract and plant colour though the family was otherwise uniform. Forty plants had red bracts and petioles palely flushed with pink; ten had yellow bracts (sometimes pinkish at the edges) and green petioles. These facts suggested that the seed from which the family was raised resulted from selfing of an isolated heterozygous plant and that the yellow character is controlled by a single recessive gene. One red and one yellow plant were self-pollinated and crossed both ways. The red-selfed and the crossed families segregated (4 red: 4 yellow and 14 red: 7 yellow respectively) and the yellow-selfed family bred true (4 yellow only). Numbers are small, but they agree with the hypothesis that yellowness is due to a single recessive gene.

This is evidence for the view that *Musa flava* Ridl. from Malaya is merely a yellow-bracted form of *Musa acuminata*. Analogous variation occurs in *Musa banksii* F. v. Muell. (Chessman, 1948), which species, however, is probably better regarded as merely another strain of the variable *M. acuminata*. Yellow bracts also occur in several cultivated banana varieties such as 'Paka' and 'Mhalihali' from Zanzibar and 'Nakawere' from Uganda (Baker and Simmonds, 1952); whether the character was derived from yellow-bracted wild ancestors or whether it originated by mutation in a red-bracted cultigen or both is not known.

Typical *Musa basjoo* Siebold was described as having 'dull brown' bracts (Cheesman, 1948) and the identification listed by Lawrence et al. (1939) of a cyanidin glycoside in the species was evidently made on such a form. The I.C.T.A. clone, however, has yellow bracts and therefore does not appear in the treatment above.

Leucoanthocyanidins

Leucoanthocyanidins (i.e. substances which yield anthocyanidins on heating with strong acid) were sought in various parts of the plant and found to be almost universally present. A survey of 13 clones belonging to 12 wild species distributed through the genus yielded the following results:

	Source.			
	Skins of fruits.	Male flowers.	Sheaths.	Petioles.
Intensity	Strong–very strong	Moderate–strong	Moderate–weak	Weak–very weak
Anthocyanidins	Dp $\gg$ Cy	Dp $\gg$ Cy	Dp $=$ Cy	Cy $>$ Dp
Number of samples	11	12	11	13

Thus the leucosubstances yielded delphinidin and cyanidin only, regardless of whether the bracts were pigmented by glycosides of these pigments or of their methylated derivatives or of pelargonidin. Flowering parts contain more of the leucoanthocyanidins than vegetative parts and the amount of delphinidin relative to cyanidin is roughly proportional to the intensity of colour developed. Thus, over all samples:

	Intensity.	
	Strong and very strong.	Moderate and weak.
Dp $>$ Cy . . .	18	9
Dp $= <$ Cy . .	0	20

for which $\chi_c^2 = 18{\cdot}9$ and $P \ll 0{\cdot}001$. Values of χ_c^2 were also calculated within each of the four sources; all showed deviations in the same direction, though only one (that for male flowers) was significant. No interpretation of these findings can be offered at present; nor is any likely to be forthcoming until the leucoanthocyanidins are better understood than they are now, both chemically and biologically.

Some seeds of various wild bananas were also examined and found to contain rather little of the leucosubstances as compared with other parts of the plant. In several samples, indeed, no anthocyanidin could be detected; when colour was developed delphinidin and cyanidin were the cause. Skin and pulp of ripe and unripe fruits of edible varieties gave intense reactions and delphinidin predominated over cyanidin, as in the skins of seeded types. Robinson (1937) detected delphinidin in the pulp of an edible banana. Bracts are also rich sources of leucoanthocyanidins, and the usual mixture (delphinidin generally predominant) was found in several acyanic types (see above). In coloured bracts their presence could be discerned by comparing chromatograms derived from hydrolysed cold 1 per cent. HCl extracts of the anthocyanins with extracts made by heating pieces of bract in strong acid; the latter always gave much greater intensities of delphinidin and cyanidin.

Comparison of the relative intensities of the various anthocyanin spots with the corresponding spots after hydrolysis suggested that the leucoanthocyanidins of the mature bract were not appreciably extracted by cold weak acid and the survey of anthocyanidins in *Musa acuminata* reported above is founded on this assumption. There are, however, leucosubstances in immature colourless bracts which undoubtedly are extracted by the same treatment and it thus appears that there is, as maturity approaches, a decline in concentration of this particular leucoanthocyanidin fraction (Simmonds, 1954*a*).

Miscellaneous observations

The anthocyanins of certain pigmented flowers, fruits, and leaves were examined. In general they had the same pigments as the bracts. Thus the pink male flowers of *Musa balbisana* contained a mixture of cyanidin and delphinidin glycosides; the red epidermis of the fruit of the Red banana and of the leaves of two vegetatively-coloured strains of *Musa acuminata* had a partially methylated mixture of pigments.

Anthocyanins in the pseudobase form were found in several parts of the banana plant. Thus colour was developed by the action of cold dilute acid on fragments (especially the margins) of unpigmented petioles, sheaths, and bracts. It was present in a minority of the plants examined and was never very strong. Traces were also found in some samples of the unpigmented male flowers of *Musa acuminata*.

One other matter, though not directly concerned with the anthocyanins, is worth mention here. Dodds and Simmonds (1948) noted that flowers of *Musa acuminata* and of F_1 hybrids with *M. balbisiana* turned brown on immersion in alcohol, whereas flowers of *M. balbisiana* itself became only slightly discoloured. Since that time it has been found that the browning reaction can be virtually stopped by plunging the flowers into boiling alcohol and that the reaction may most easily be demonstrated by grinding flowers in water and examining a suspension of the debris in a test-tube. Addition of catechol to an extract of flowers of *Musa balbisiana* caused rapid browning. It therefore seems likely that the two species both contain a polyphenol oxidase system but that flowers of *M. acuminata* alone contain substrates that can be condensed by the system to visible brown quinonoid complexes; flowers of *Musa balbisiana* lack a substrate and therefore do not turn brown. This would seem to imply that the oxidase system cannot act on the ortho- and para-diphenolic groupings of the anthocyanins, leucoanthocyanidins, and anthoxanthins (that are present in the flowers) to produce perceptible brown colours.

DISCUSSION

The anthocyanins of the bananas are characterized as a whole by the complexity of their anthocyanidins and the simplicity of their glycosidic combinations. Of the known anthocyanidins only hirsutidin and gesneridin have not been found in the genus and, of the remaining six, no fewer than five may occur in a single bract. Pigment chemistry has considerable taxonomic value and it is likely that an exact knowledge of the anthoxanthins would enable us to identify all or at least nearly all the wild species by their flavonoid pigments alone. Particularly interesting is the difference between *M. acuminata* with partially methylated pigments and the undescribed Assam species called here 'Mariani', with fully methylated anthocyanins. 'Mariani' resembles *M. acuminata* phenotypically, but in breeding behaviour is at least as closely related to members of the section *Rhodochlamys* (unpublished). Thus its anthocyanin chemistry supports the genetical view that it is specifically distinct from *M. acuminata.* Contrariwise, one of the few instances in which study of two wild species has not revealed a difference between them is instructive for this very reason (*M. violascens* and *M. ornata* referred to above). Among the cultivated bananas, the flavonoid pigments provide useful evidence of the nature of the type of the Linnaean species *M. paradisiaca*, a matter of fundamental importance for the nomenclature of the edible bananas as a whole.

The bananas would seem to offer favourable material for the study of two questions of more general biological and chemical interest—the structure and function of the leucoanthocyanidins and the biogenesis of the flavonoid pigments. The leucoanthocyanidins of bananas, it will be noted, follow the general rule proposed by Lawrence et al (1939), that they are synthetically simpler than the corresponding visible pigments. The male bud of the banana (by reason of the seriation and comparatively large size of the bracts) offers peculiarly favourable material for investigation of the second problem, as has been pointed out elsewhere (Simmonds, 1954*a*). Possible approaches would be: the search for precursors of the kind found by Birch, Donovan, and Moewus (1953), comparison of anthocyanin and anthoxanthin structures (Geissman and Mehlquist, 1947), and the use of 'feeding experiments' on genetically acyanic varieties (cf. the elegant and suggestive observations of Robinson, 1939, on *Arnebia*).

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The Laminariaceae off North Shapinsay: Changes from 1947 to 1953

BY

F. T. WALKER

(*Institute of Seaweed Research, Musselburgh, Midlothian, Scotland.*)

With six Figures in the Text

ABSTRACT

Four sublittoral surveys, off the north coast of the island of Shapinsay, Orkney, have been completed in May 1947, July 1951, July 1952, and July 1953.

The calculated quantity of seaweed from 1 to 6 fathoms was 34,000 tons during the initial survey of May 1947 after a comparatively mild winter and before the period when bad weather brings about the loss of large quantities of the previous year's growth of laminaria fronds. The three resurveys were completed after this.

The quantity of seaweed in July 1951 was 10,000 tons, and in July 1952 a further reduction was found, bringing down the quantity to 6,000 tons. In July 1953 the quantity had partially recovered, reaching 7,000 tons. The increase was reflected more in the seaweed cover than in density. Between 1952 and 1953 the weight of individual plants had decreased but the number of plants per unit area was greater, which, combined with an increase in the area of seabed covered, made an overall gain in quantity. *Laminaria saccharina* maintained its dominance in this area throughout the period 1947–53.

INTRODUCTION

AS part of the programme of ecological research, whose object it is to record perennial and cyclic changes which may occur to extensive areas of algae growing in the sublittoral zone off the coast of Scotland, Shapinsay was selected as one location representative of *Laminaria saccharina*. (The Isle of Arran in the Firth of Clyde is another location.)

The island of Shapinsay lies north-north-east of Kirkwall, the chief town and port of the mainland of the Orkney Islands. The survey area lies off the northern coast of Shapinsay between Galt Ness and Sandy Geo and includes the extensive Veantrow Bay, Fig. 1. The surface of the seabed in this area is one of rocks, boulders, and sand. The characteristic formation of Orkney is Old Red Sandstone. The mean tidal range is 1 fathom (2 m.), and during spring tides the rate of tidal stream passing the north-west coast of Shapinsay is 6–7 knots.

The survey area was first observed in 1946 from the air and later studied from aerial photographs taken by the Royal Air Force and loaned by the Department of Health, to whom acknowledgement is made for their co-operation. The preliminary survey showed the area to carry a high percentage of seaweed cover, and on this evidence the first detailed survey (sampling by

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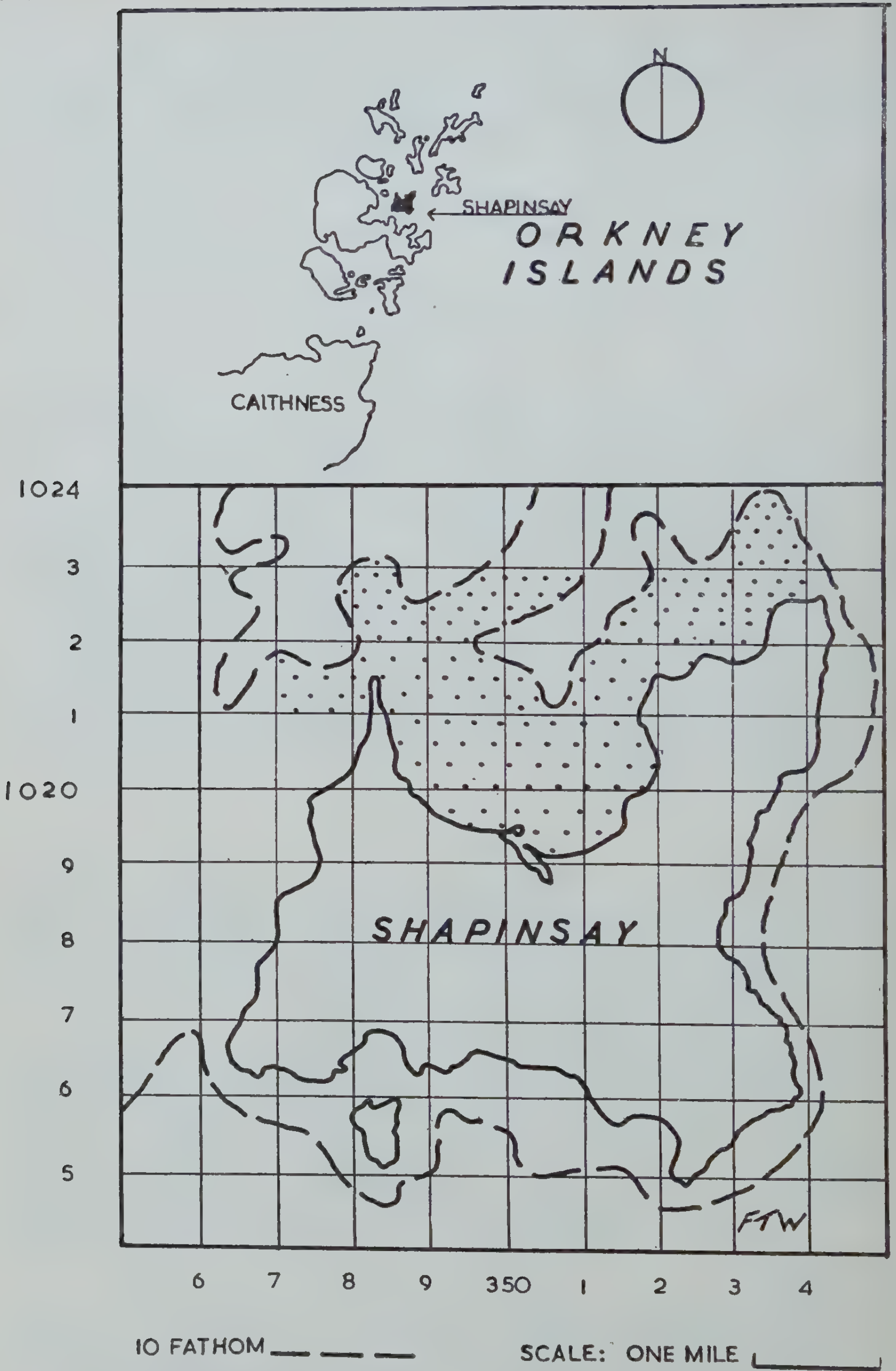

FIG. 1. Map of Shapinsay, Orkney, based on Ordnance Survey map. The lines are those of the kilometre National Grid. The squares containing dots cover the area sampled at random with an intensity of 250 quadrats/km.² between 0 and 10 fathoms, L.W.M.O.S.T.

calibrated spring-grab from a motor vessel) was carried out in May 1947 (Walker, 1950). Three resurveys each during the month of July were made in 1951, 1952, and 1953.

METHODS

The 1947 survey employed quadrat sampling at fathom intervals along transects measured by a rangefinder; the transects were 200 yards apart (Walker, 1947). The three resurveys employed random sampling with an intensity of 250 quadrats per square kilometre from 0 to 10 fathoms; a total of 10,988 quadrats were measured in the four surveys.

Quadrats were taken at all times, but chiefly on the flood tide, particularly in shallow water.

All depths were recorded to the nearest fathom (2 metres). All depths have been standardized to read L.W.M.O.S.T. 'Density' given in this paper represents the fresh weight per unit area of the algae immediately they were taken from the sea. The weighing was to the nearest half-pound using a spring balance.

TABLE I

Calculated Total Seaweed off the North Coast of Shapinsay covering 1,040 Acres of the Sublittoral Zone between 1 and 6 Fathoms (Inc.) L.W.M.O.S.T., based on 7,600 Quadrats

Date.	Total seaweed, fresh weight (tons).	Cover (%).	Mean density, fresh weight (tons/acre).	Standard Error of density mean (%).
May 1947 . . .	33,960	78	32·6	—
July 1951 . . .	9,890	52	9·5	2 (3)
July 1952 . . .	5,830	35	5·6	3 (4)
July 1953 . . .	6,860	48	6·6	3 (3)

'Cover' where quoted is the number of quadrats which contained some seaweed expressed as a percentage of all quadrats.

For easier comparison the results have been represented graphically rather than in tables.

The first survey in May 1947 was limited to depths from 1 to 6 fathoms for economic reasons, while the resurveys were carried out from 0 to 10 fathoms; therefore, in order to make comparisons between the four surveys, the data of each have been presented to cover depths of 1 to 6 fathoms only in Table I and Figs. 2 and 4.

The mean densities in Table I are master means calculated from means found at depths of 1 to 6 fathoms. The standard error of the mean density has been calculated by the method of pooled variances found at various depths in the case of the three resurveys when random sampling was employed. The standard error for all quadrats irrespective of depth is given (in brackets) for comparison.

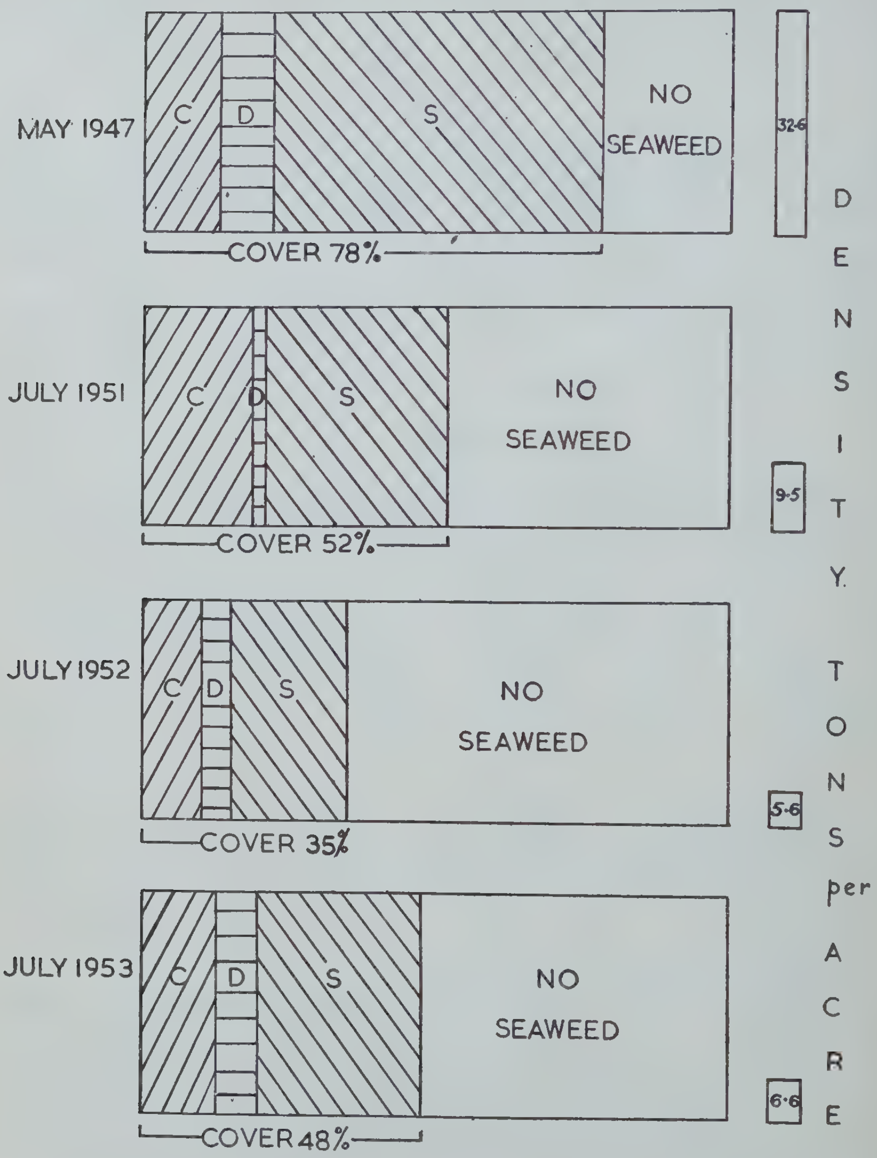

FIG. 2. North Shapinsay, Orkney. Diagrammatic representation of changes in the seaweed cover, density, and ratio of species (based on the fresh weights) over the area from 1 to 6 fathoms inclusive from May 1947 to July 1951 to July 1952 to July 1953. C, *Laminaria cloustoni*; D, *L. digitata*; S, *L. saccharina.*

RESULTS

The calculated total quantity of seaweed and the relative cover for each of the four surveys from 1 to 6 fathoms are given in Table I.

The density, cover, and ratio of species of laminariae which constitute the bulk of the seaweed are represented in Fig. 2.

The mean density of seaweed and the respective cover found at fathom intervals of depth for each of the four surveys are given in Table II.

TABLE II

Depth fathoms, L.W.M.O.S.T.	Mean density, based on total quadrats, fresh weight (lb./sq. yd.).				Cover (%).			
	May 1947.	July 1951.	July 1952.	July 1953.	May 1947.	July 1951.	July 1952.	July 1953.
0	*	10·4	5·2	4·2	*	73	41	48
1	18·2	9·6	4·7	5·1	81	75	42	52
2	19·3	6·4	3·2	4·7	79	59	39	52
3	15·7	3·6	2·6	2·9	78	50	36	47
4	16·0	2·3	1·8	2·4	80	43	31	48
5	11·0	2·5	1·8	1·7	70	47	36	43
6	10·3	1·8	1·4	1·5	70	44	33	48
7	6·8	1·4	0·8	1·0	65	34	23	38
8	*	0·6	0·5	0·7	*	24	19	32
9	*	0·3	0·4	0·5	*	12	18	26
10	*	0·7	0	0·3	*	20	0	21
Whole survey as unit, 0–10 fathoms (inc.) .	—	4·2	2·0	2·2	—	50	31	42

* No quadrats taken.

The mean density of seaweed at various depths, based on (1) all quadrats, irrespective of whether laminariaceae was or was not brought up, and (2) on quadrats which contained these algae, are plotted on logarithmic scales in Fig. 3.

The relationships of seaweed density and cover are given in Fig. 4.

The ratios of species at intervals of depth for the four surveys are represented in Fig. 5.

The coefficients of variability at intervals of depth for three resurveys when random sampling was employed are plotted in Fig. 6.

In the course of the 1952 and 1953 surveys the number of plants/quadrat was recorded, making possible the complication of Table III.

DISCUSSION

It was found from the results of the 1947 detailed survey that the seaweed cover was 78 per cent. and the same value was measured from aerial photographs taken 2 years later. It would appear that stability of the cover might be found, but in July 1951 the cover decreased to 52 per cent. and again in July

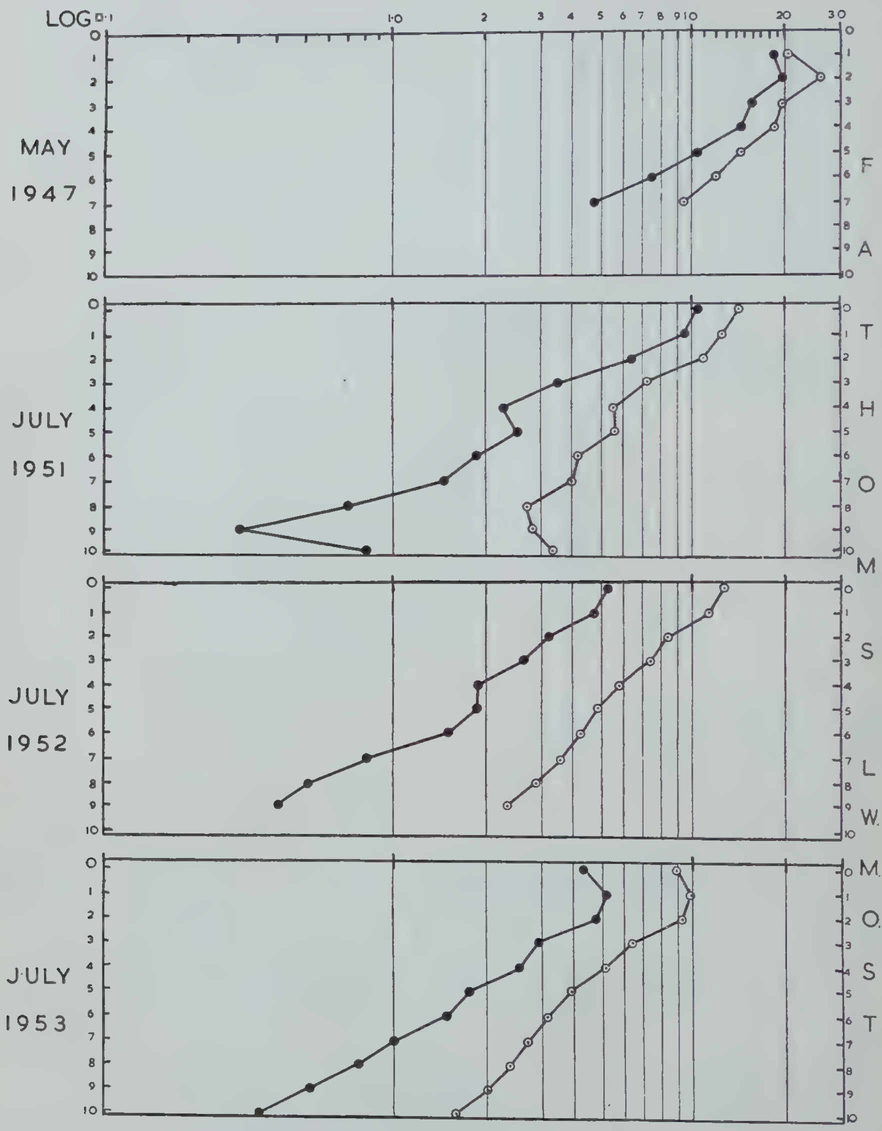

FIG. 3. Mean densities of seaweed at fathom intervals of depth calculated from all quadrats (dots) and from quadrats containing Laminariae (circles). The results are plotted on a logarithmic scale ($\log_{10}$).

1952 to 35 per cent.; however, in July 1953 it had recovered to 48 per cent., almost that of 1951.

The mean seaweed density (based on all quadrats, irrespective of whether or not they contained seaweed, and therefore including the factor of cover)

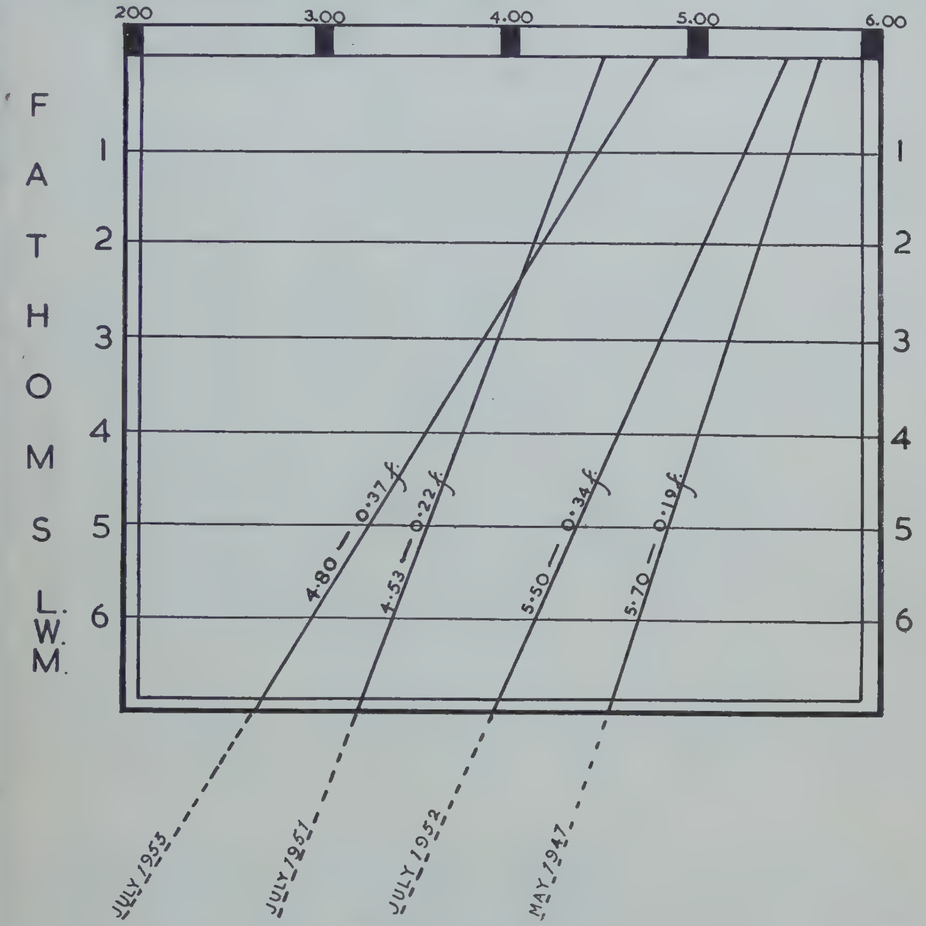

FIG. 4. The relationship of seaweed density and cover at fathom intervals of depth from 1 to 6 fathoms for May 1947, July 1951, July 1952, and July 1953. The values are found from the square root of the density (lb./sq. yd.) divided by the cover (%).

was 32·6 tons/acre in 1947, a high value compared with other areas supporting Laminariaceae. In 1951 the density decreased to 9·5 tons/acre and again in 1952 to 5·6 tons/acre, but in 1953 it recovered to 6·6 tons/acre, but this increase was not to the same degree as that of the cover (Figs. 2 and 3).

The above findings were found to apply at individual depths (Table II).

Two points must be borne in mind when comparing the 1947 survey with the three later surveys of this area. Firstly, that the 1947 survey was completed before the occurrence of severe weather known in Orkney as the 'gab of

May' and the 1951, 1952, and 1953 surveys were started after this phenomenon. Secondly, if the winter storms are relatively mild and so permit large and mature plants of the pseudo-annual *Laminaria saccharina* to survive into the following year and the perennial *Laminaria cloustoni* to remain attached to the substrata, then the presence of these large plants will increase the seaweed density and maintain the cover until the 'gab of May' takes toll.

TABLE III

Average Number of Plants in One Square Yard and Average Fresh Weight of Plants (without Holdfast) of the Chief Species of Laminariaceae found at Various Depths

Depth fathoms L.W.M.O.S.T.	*Laminaria cloustoni.*				*Laminaria digitata.*				*Laminaria saccharina.*			
	Average No. of plants in square yard.		Average weight of plant (lb.).		Average No. of plants in square yard.		Average weight of plant (lb.).		Average No. of plants in square yard.		Average weight of plant (lb.).	
	1952	1953	1952	1953	1952	1953	1952	1953	1952	1953	1952	1953
0 . . .	6·1	7·1	2·2	1·2	4·0	5·0	2·3	1·1	8·0	4·9	1·0	0·6
1 . . .	5·6	7·6	2·0	1·2	4·9	4·9	2·1	2·0	7·8	5·0	1·0	0·6
2 . . .	4·6	10·6	2·5	1·0	6·0	5·4	1·8	2·1	4·8	6·7	1·0	0·7
3 . . .	5·3	7·9	2·3	1·3	7·3	5·3	2·0	2·7	6·3	7·5	0·9	0·6
4 . . .	—	—	—	—	—	—	—	—	6·5	6·8	0·8	0·6
5 . . .	—	—	—	—	—	—	—	—	5·6	6·3	0·8	0·5
6 . . .	—	—	—	—	—	—	—	—	4·9	5·7	0·8	0·5
7 . . .	—	—	—	—	—	—	—	—	3·8	4·7	0·8	0·5
8 . . .	—	—	—	—	—	—	—	—	3·3	4·2	0·8	0·5
9 . . .	—	—	—	—	—	—	—	—	2·9	4·1	0·8	0·5
10 . . .	—	—	—	—	—	—	—	—	—	3·5	—	0·6
	0–3 fathoms inc.				0–3 fathoms inc.				0–9 fathoms inc.			
1952 . .	308 plants, mean wt. 2·2 lb.				150 plants, mean wt. 2·0 lb.				2,086 plants, mean wt. 0·84 lb.			
1953 . .	391 plants, mean wt. 1·1 lb.				84 plants, mean wt. 1·9 lb.				3,160 plants, mean wt. 0·56 lb.			

Surveys of cast weed, carried out during the winters of 1945–6 and 1946–7 in various parts of Scotland, showed the quantities in 1946–7 were half those found in 1945–6. These results suggest that the winter of 1946–7 was less damaging to the laminaria than the previous winter.

The mature plants of *L. cloustoni* which survive the winter lose their old fronds during the 'gab of May'. Such seaweed is known as the 'May cast'. The mature plants of *L. saccharina* which survive the winter may also be cast during the same period. The greater part of this species is composed of frond.

Where the frond of *L. cloustoni* is cast during May the seaweed density decreases but the cover is little affected, whereas when *L. saccharina* is cast both the density and the cover decrease.

The relationship of density and cover at various depths for the four surveys is plotted in Fig. 4. This relationship, which has been found to hold in many

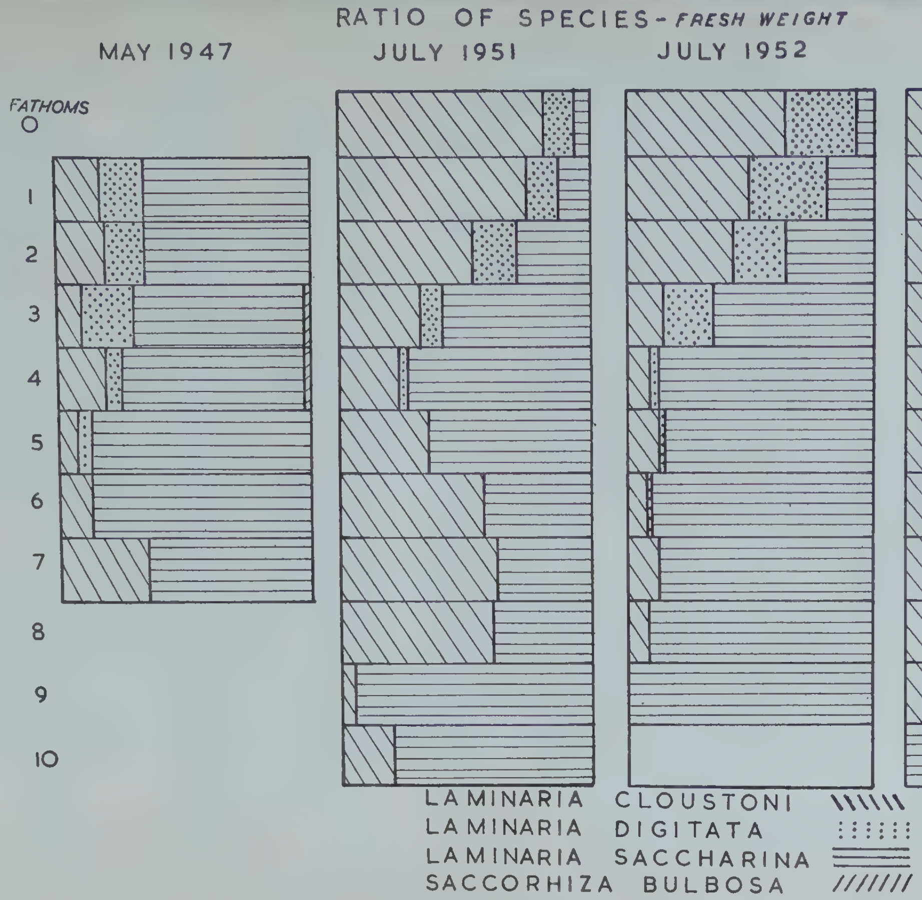

FIG. 5. Ratio of species at various depths (based on fresh weight) found during May 1947, July 1951, July 1952, and July 1953.

sublittoral surveys off the coast of Scotland (Walker, 1950, 1953), conforms to the formula $\sqrt{d}/C = k-\gamma f$,

where d = mean density of seaweed based on all quadrats, for various intervals of depth,
C = cover at various intervals of depth,
f = interval of depth,
k = a constant for the survey,
γ = a constant of regression with increasing depth.

The movement of the graph to the right or left shows the change in the *relationship* between plant growth and algal cover.

As the cover increased at a greater rate than the density in 1953 compared with 1952, we might expect the number of plants/unit area to show a greater increase than the mean weight of individual plants. This is confirmed by the results given in Table III, where it will be found that smaller plants are covering a larger area; recolonization is taking place. It is also apparent that the number of plants/unit area varied with depth to a greater degree than the mean weight of individual plants. Decrease in seaweed density (Fig. 3) is caused more by the reduction in the number of plants than in their individual weight. This has been reported previously (Walker, 1948, 1950); nevertheless the weight of the mature plants of *L. saccharina* in 1947 was six times that found in 1953 (the plants of 1947 were too large to be the product of one season's growth). The total seaweed in 1947 was five times that of 1953 (Table I).

While *Laminaria saccharina* has maintained its dominance over the area, there have been changes in the ratio of the three major species. In 1951 *Laminaria cloustoni* was well established, but during 1952 and 1953 had lost ground, particularly in the deeper water, giving place to *L. saccharina* and to a less degree in the shallower water to *L. digitata* (Fig. 5).

One disadvantage of comparing averages is the absence of information regarding the frequency and spread of the samples contributing to the average. The standard deviation gets over this, but then we are faced with a comparison of various means with differing deviations. A factor for easier comparison is the *coefficient of variability*, obtained by dividing the standard deviation of the mean by the mean. The coefficients of variability, ranging from 35 to 86 of all quadrats which contained laminariae, for each depth (0–10 fathoms L.W.M.O.S.T.) for the surveys of 1951, 1952, and 1953 are plotted in Fig. 6.

The range of the coefficients of variability is the same as that obtained from the data of nine surveys in three areas of Scotland where *Laminaria cloustoni* was the dominant species (presented for publication).

The overall picture visualized by the foregoing surveys is that from 1947 to 1949 the weed bed enjoyed stability and conditions for plant survival throughout the winter. Between 1949 and 1951 a large part of the weed bed was destroyed, and this continued into 1952. By 1953 recolonization had become evident. A further survey is recommended in July 1955 or 1956 provided the intervening winters are not severe at sea.

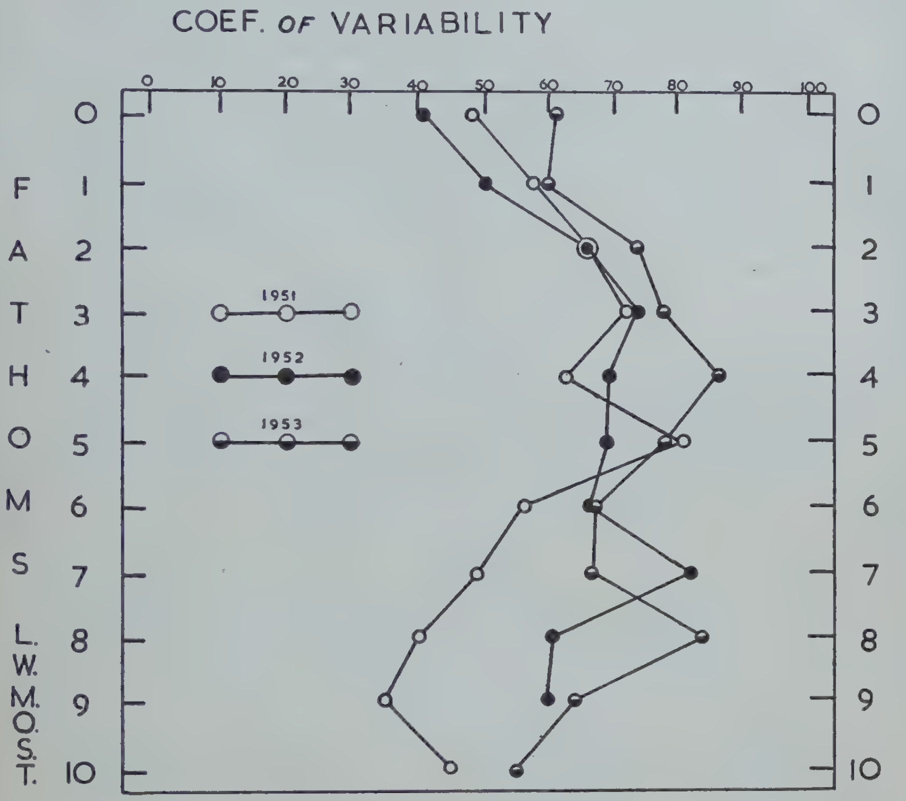

FIG. 6. Coefficients of variability of quadrats containing Laminariae, at fathom intervals of depth, for surveys carried out during July 1951, July 1952, and July 1953. Range 35–86. (Coefficient of variability is obtained from the standard deviation and the mean.)

Note in proof

Since this paper was presented for publication I have found that the product of the seaweed cover (expressed as a percentage in Table I, col. 3) and the square root of number of hours of duration of STRONG GALES in the year, July–June, preceding each survey respectively (recorded at Lerwick, Shetland, and published by the Meteorological Office) is a constant. In other words, the seaweed cover is inversely proportional to the square root of the duration of strong gales, as defined by the Meteorological Office, in the Orkney–Shetland area. This finding indicates a useful line of research.

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I wish to put on record the conscientious work of sampling the seabed off the coast of North Shapinsay by the crew of the m.v. *Teresa* and the assistance of W. D. Richardson, B.Sc., who joined my staff in 1952.

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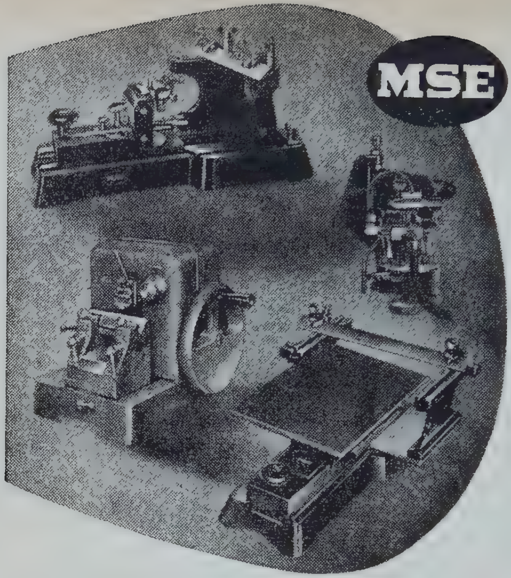

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